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Index

No. 1

Wickramasinghe, S. N. and Bush, V. Electron Microscope and High Resolution Autoradiographic Studies of Megaloblastic Erythropoiesis 1

Bitran, J., Golomb, H. M., and Rowley, J. D. Idiopathic Acquired Refractory Sideroblastic Anemia. Banded Chromosome Analysis in Six Patients 15

Baumgartner-Stäubli, R. and Beck, E. A. Sideroblast Score. A Sensitive Indicator of Iron Deficiency and Hypoproliferative Anemia 4

Galanello, R., Melis, M. A., Muroni, P., and Cao, A. Quantitation of Hb A_2 with DE-52 Microchromatography in Whole Blood as Screening Test for β-Thalassemia Heterozygotes 32

Dachà, M., Canestrari, F., Bossù, M., Rossi Ferrini, P. L., and Fornaini, G. Inherited Erythrocyte Pyruvate Kinase Deficiency: Studies on 15 Members of Two Related Families 37

Stathakis, N. E., Papayannis, A. G., Antonopoulos, M., and Gardikas, C. Familial Thrombosis Due to Antithrombin III Deficiency in a Greek Family 47

Foadi, M. D. Tetraploid Cell Line in a Girl with Acute Leukaemia 55

No. 2

Kanakoudi-Tsakalidou, F., Spyridolou, K., Tzafi, R., and Cassimos, C. Effect of Blood Transfusion on the Immune Response of Children with Thalassaemia 65

Winterleitner, H., Rotter, M., and Knapp, W. A Simplified One-Step Procedure for the Simultaneous Determination of Complement Receptor Lymphocytes and Lymphocytes with Membrane-Bound Immunoglobulins 74

Iyengar, B. Histogenesis of Myeloid Metaplasia in the Spleen 81

Rafflenbeul, D. and Zbinden, G. Circulating Platelet Aggregates and Thrombocytopenia Induced by Intravenous Infusions of Arachidonic and Lauric Acids in Guinea Pigs 87

Westerhausen, M. and Meuret, G. Transferrin-Immune Complex Disease 96

SANSONE, G SCIARRATTA, G V GENOVA, R. DARBRE P D., and LEHMANN, H Haemoglobin Shepherds Bush (β74 [E 18] Gly→Asp) in an Italian Family 102
GANESAN J LIE-INJO, L. E. NG, T S. and GEORGE, R. Interaction of Haemoglobin E with α- Thalassaemia and Haemoglobin Constant Spring 109
KENDALL, A. G and CHARLOW G F Red Cell Pyruvate Kinase Deficiency Adverse Effect of Oral Contraceptives 116
MANDELLI F AMADORI, S. DE LAURENZI, A. KAHN A. IACCHI G and PAPA, G Glucose-6-Phosphate Dehydrogenase Velletri. A New Variant with Reduced Activity in a Patient with Congenital Non-Spherocytic Haemolytic Anaemia 121

No. 3

DOUGLAS, S. D and OOKA, M P Freeze-Fracture of Circulating Human Eosinophils 129
GANDOLFO G M AFELTRA, A AMOROSO, A. BIANCOLELLA, F and FERRI, G M Circulating Anticoagulant against Factor XII and Platelet Antibodies in Systemic Lupus Erythematosus 135
GROVE S. S. JENKINS, T KAMUZORA, H L. and LEHMANN, H. Congenital Heinz Body Haemolytic Anaemia Due to Haemoglobin Perth in a Nama Child Seemingly Aggravated by the High Nitrate Content of the Water Supply 143
DAVIDSON, R J L. and LESSELS, S. E. Infectious Mononucleosis in Hodgkin's Disease. A Further Case Report 152
GORST D W COURTIS, M., and DELAMORE, I W A Study of Folic Acid-Binding Protein in Normal Subjects 156
TILLMANN, W GAHR, M LABITZKE, N and SCHRÖTER, W Membrane Deformability of Erythrocytes with Glucose-6-Phosphate Dehydrogenase Hamburg 162
CRISTER, D RUBINSTEIN I and MENACHE, R. A Simple and Rapid Platelet Calcium Determination. Normal Values and Results in May Hegglin Anomaly 168
KELEMEN, E. KRASZNAI, G ENDES, P and SZINAY G Chronic Idiopathic Myelofibrosis. A Reversible Disease? 171
BALDUINI C. L. RICEVUTI, G SOMMO M C. ASCARI E. BROVELLI A., and BALDUINI C. *In vivo* Behaviour of Neuraminidase-Treated Rabbit Erythrocytes and Reticulocytes 178
ACAR, S. TEKINALP G ÖZSOYLU Ş. ÇEVIK N and YAŞAR, H Burkitt's Lymphoma Cell Leukemia 188

No. 4

VETTORE, L. DE MATTEIS, M. C. and DEL CONTE G Effects of Dipyridamole on Sodium and Potassium Content of Human Red Blood Cells 193
MICHALEVICZ, R. MANY A., and RAMOT B Peripheral Blood Lymphocytes in Systemic Lupus erythematosus- Relation to Activity 199
GREALLY J F WHELAN, C A. O'CONNELL, L. and O'GORMAN, D Cold Agglutinins in a Case of Chronic Lymphatic Leukaemia A Study of the Lymphocyte Surface 206

CARELLA, A. M. BACIGALUPO, A. SANTINI, G. GIORDANO, D DAMASIO, E. E. PUSCO, F A. ROSSI, E., and MARMONT A. M. *Peptichemio* A New Oncolytic Drug in Combination with Vincristine and Prednisolone in the Treatment of Non-Hodgkin Lymphomas 211

POMARI, O. CIVARDI, E. MICHELA, A. PINI, M. POTI, R., and DETTORI, A. G., Inherited Platelet Abnormalities Associated with Low Factor VIII Activity in the Same Family 225

GREENBERGER, J S. BOCACCINO, C. A. SCOTT S. J and MOLONEY W C. Chemotherapeutic Remissions in Wistar Furth Rat Acute Myelogenous Leukemia A Model for Human AML 233

KUBISZ, P PAŘIZEK, M. KOVÁČOVÁ, J and CRONBERG, S., Influence of Cytostatics on Some Platelet Functions *in vitro*. V Hydroxyurea 42

PISCHEL, K. CORDONI, G. SZAMSAWSKI, W and BUTTINONI, G Scanning Electron Microscopic Study of Leukemic Human B Lymphocytes 247

No. 5

PANDOLFI, F LUZI, G. TOZZI, M. C., and AIUTI, F Preparation, Purification and *in vitro* Properties of a Serum against Human Lymphoblastic Leukemia-Associated Antigens 257

ECONOMOPOULOS, T C. PAPAYANNIS, A. G STATHAKIS, N. E. ARAPAKIS, G., and GARDIKAS, C. Postoperative Platelet Function in Patients on Small Subcutaneous Doses of Heparin 266

KLENER, P and DONNER, L. Imidazole Carboxamide (DTIC) in the Treatment of Advanced Lymphomas Efficacy of DTIC in Cases which Fail to Respond to Conventional Chemotherapeutic Combinations 272

ROZENSZAJN, L. A. DAVID, E. R., and SELA, S B Large Granules and Lysosomal Fusion in Human Chediak-Higashi White Blood Cells 279

BAELE, G MATTHIJS, E., and BAREZZ, F Antihaemophilic Factor A Activity F VIII-Related Antigen and on Willebrand Factor in Hepatic Cirrhosis 290

GRONBLA J MAMMON, Z. LEWITUS, Z., and JOSHUA, H. Chronic Myelogenous Leukemia with Elevated Leukocyte Alkaline Phosphatase, Positive Indirect Coombs Test, Neutrophilic Leukocytosis and Unusual Cytogenetical Findings 298

CHALEVELAKIS, G. LYBERATOS, C. MANOPOULOS, D PYROVOLAKIS, J and GARDIKAS, C. Erythrocyte δ-Aminolaevulinic Acid Dehydratase Urinary Porphyrins and Porphyrin Precursors in Iron Deficiency Anaemia 305

DILEO, P E. MÜLLER, H. OBRECHT, J-P SPECK, B. BÜHLER, E. M and STALDER, G R. Loss of the Y Chromosome from Bone Marrow Cells of Males with Myeloproliferative Disorders. Report of Two Cases and Review of the Literature 310

No. 6

HUSZ, S. KELEMEN, F DOBOZY A. HUNYADI, J and SIMON, N. B and T Cell Markers of Bone Marrow and Peripheral Blood Lymphoid Cells in Patients with Paraproteinaemia 321

Haake, R. and Robinson, E. Lymphocyte Reactivity in Healthy Subjects and Cancer Patients 331
Helmstädter, V., Arnold, H., Blume, K. G., Uhl, N. and Hunstein, W. Acquired Pyruvate Kinase Deficiency with Hemolysis in Preleukemia 339
Invernizzi, F., Pioltelli, P., Gavazzeni, G., Riboldi, P. S., and Zanussi, C. Anti-γ-Globulin Activity, DNA and Antibodies to DNA in Nonlupoid Cryoglobulinemias 344
Dash, S. and Bhagwat, A. G. Combined G-6PD and 6-PGD Deficiency in a Hindu Boy 351
Penchas, S., Zlotnick, A., Cohen, R. and Cohen, M. M. Pyridoxal-5-Phosphate-Resistant Sideroblastic Anaemia with Trisomy 8 Mosaicism in the Bone Marrow 354
Kozuru, M., Benoki, H., Sugimoto, H., Sakai, K. and Ibayashi, H. A Case of Lambda Type Tetramer Bence-Jones Proteinemia 359

In Memory of Professor Heinrich Lüdin 366

Book Reviews · Buchbesprechungen · Livres nouveaux 127

Index rerum 369
Index autorum 383
Index Vol. 57 after · nach · après 386

Acta haemat. 57 1 14 (1977)

Electron Microscope and High Resolution Autoradiographic Studies of Megaloblastic Erythropoiesis

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Key Words. Autoradiography DNA synthesis Electron microscopy Folate deficiency Megaloblasts Protein synthesis RNA synthesis Vitami B_{12} deficiency

Abstract Bone marrow fragments from 10 patients with megaloblastic anaemia due to vitamin B_{12} or folate deficiency were studied by electron microscopy and electron microscope autoradiography A proportion of the erythroblasts showed ultrastructural abnormalities. Some of the cells containing autophagic vacuoles, large siderosomes, iron-laden mitochondria, irregularly shaped nuclei, membrane-bound nuclear clefts, or incomplete nuclear membranes were found to be capable of DNA, RNA and protein synthesis. Other cells showed advanced degenerative changes such as the distension of the perinuclear space, the clumping of cytoplasmic organelles near the nucleus and reduction in the electron density and ribosome content of the cytoplasm. Most of these grossly abnormal cells suffered from either marked depression or an arrest protein and RNA synthesis, and were presumably destined for phagocytosis by reticulum cells.

Although several authors have commented on various ultrastructural abnormalities which affect the erythroblasts of patients with megaloblastic haemopoiesis [5 9 17] there have been few detailed reports on the ultrastructure of megaloblastic erythropoiesis. In this paper we describe the abnormalities detected during an electron microscope study of the erythroblasts of patients with megaloblastic haemopoiesis due to vitamin B_{12} or folic acid deficiency In addition, we present autoradiographic

data on the metabolic activity of the cells showing some of these ultra structural abnormalities

Patients and Methods

Ten patients with a megaloblastic anaemia were studied 4 of these were vitamin B_{12}-deficient (Hb 6.0–8.7 g/dl MCV 109–170 fl) and the remainder were folate deficient (Hb 4.9–9.3 g/dl MCV 105–142 fl) Marrow was aspirated from the sternum and a portion of the aspirate was immediately mixed in about 5 ml of Hanks solution containing preservative-free heparin A few marrow fragments were removed from this marrow suspension, using a Pasteur pipette, and these fragments were fixed in a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), usually for –4 h.

A ml aliquot of the marrow suspension was incubated with 40 μCi of 6-^{3}H thymidine (^{3}H TdR specific activity 26 Ci/mmol) in 1 patient with vitamin B_{12} deficiency (Hb 6.7 g/dl MCV 106 fl) and 1 patient with folate deficiency (Hb 4.9 g/dl MCV 142 fl) with 170 μCi of L-4,5-^{3}H leucine (specific activity 58 Ci/mmol) in 1 patient with vitamin B deficiency (Hb 7.0 g/dl MCV 1 0 fl) and 2 patients with folate deficiency (Hb 6.9 and 9.3 g/dl MCV 105 and 109 fl), and with 100 μCi 5-^{3}H uridine (specific activity 24 Ci/mmol) in 1 patient with vitamin B_{12} deficiency (Hb 7.0 g/dl MCV 120 fl). All incubations were carried out at 37 C for 1 h, after which a few small labelled fragments were fixed in glutaraldehyde, as before

The glutaraldehyde-fixed marrow fragments were washed in 0.1 M phosphate buffer post fixed in buffered 1 osmium tetroxide for 3 h, dehydrated in ascending grades of alcohol embedded in Araldite which was polymerized at 60 C for 7 h and sectioned on an LKB Ultrotome III using a glass knife. In the case of the non radioactive tissue, the sections (60-90 nm thick) were mounted on uncoated copper grids, sequentially stained in uranyl acetate and Reynold's lead citrate, and examined in an AEI 6B electron microscope

For the preparation of electron microscope autoradiographs, the unstained sections were mounted on collodion-coated slides, vacuum-coated with carbon and covered with a monolayer of Ilford L4 emulsion. The autoradiographs were exposed for 4–10 weeks, developed either with ID19 or with gold latensification and Elon ascorbic acid [15] and fixed in 25% sodium thiosulphate. The collodion membrane was then stripped off the slide and the sections together with the overlying autoradiographs were attached to grids for staining and examination under the electron microscope.

Results

The electron microscope findings were qualitatively similar in all 10 patients with megaloblastic haemopoiesis, but the frequency with which various abnormalities were found varied considerably from patient to patient.

Ultrastructure of Megaloblastic Erythropoiesis

Basophilic megaloblasts. These cells possessed a large, more or less rounded nucleus with little or no peripheral chromatin condensation and one or more nucleoli. Their cytoplasm contained numerous ribosomes (mostly arranged as polysomes), a moderate number of mitochondria, and unusually long strands of rough endoplasmic reticulum. Some cell profiles contained annulate lamellae, a Golgi apparatus or a small group of pleomorphic cytoplasmic granules. The longest strands of endoplasmic reticulum coursed through half or more of the cell profile. An occasional profile contained several short strands of endoplasmic reticulum stacked parallel to each other or contained one or two more or less circular strands of endoplasmic reticulum which completely isolated portions of the cytoplasm within them.

Polychromatic megaloblasts. The maturation of the basophilic megaloblasts into non-dividing late polychromatic megaloblasts was accompanied by (1) a progressive increase in the electron density of the cytoplasm (presumably due to a progressive increase in its haemoglobin content), (2) a progressive increase in the amount of heterochromatin within the nucleus, and (3) a decrease in the number of ribosomes and mitochondria within the cytoplasm. These changes are qualitatively similar to those observed during the maturation of normal basophilic erythropoietic cells into late polychromatic normoblasts. However, the amount of heterochromatin present in some or all of the early and late polychromatic cells appeared to be smaller in megaloblastic marrow than in normal marrow. In addition, some polychromatic megaloblast profiles showed various other ultrastructural abnormalities. These abnormalities, which are summarized in table I, were more frequently seen in the late (non-dividing) polychromatic megaloblasts than in the early (dividing) polychromatic megaloblasts. Some of the abnormalities listed in table I are illustrated in figures 1–4 and are dealt with in greater detail below.

A proportion of the circular cytoplasmic membranes present within the polychromatic megaloblasts resembled those seen within the basophilic megaloblasts, apart from the fact that they sometimes did not show membrane-associated ribosomes (fig. 1a, c). The regions of cytoplasm isolated by such membranes varied in area and shape, were sometimes partially digested (i.e. showed a decreased electron density), and occasionally contained myelin figures (fig. 3a), ferritin particles, degenerating mitochondria or several small vesicles. At least some of these membrane-bound zones probably represent autophagic vacuoles. Another

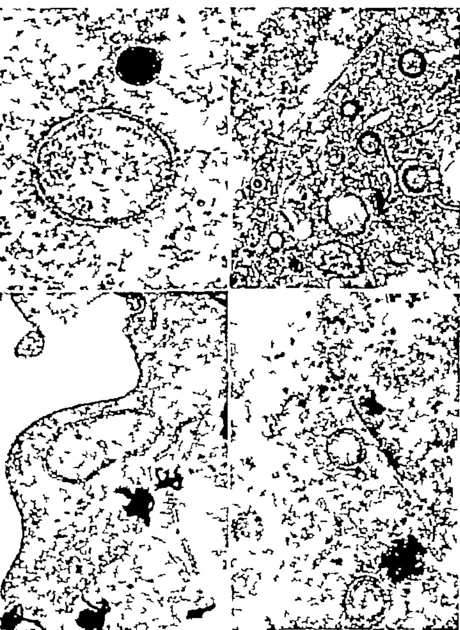
type of presumably autophagic vacuole encountered within the polychromatic megaloblasts was composed of 2 concentric layers of membrane separated from each other by electron-dense material (fig. 1b d) The inner membrane appeared to enclose a portion of the cytoplasm within it. The cytoplasm of some of the polychromatic erythroblast profiles contained inclusions consisting of nuclear material Most of these inclusions were bounded by a double membrane and consisted either of con densed chromatin only or of both condensed and expanded chromatin (fig. 2a) A few of the inclusions were not surrounded by any membrane and consisted entirely of condensed chromatin (fig. 2b)

Bone marrow reticulum cells These cells contained one or more de generating erythroblasts erythrocytes and granulocytopoietic cells within their cytoplasm. In general the phagocytosed erythroblasts showed more advanced degenerative changes than the erythroblasts which lay outside the reticulum cells

Electron Microscope Autoradiographs

The electron microscope autoradiographs of marrow fragments la belled with ^{3}H TdR showed that basophilic megaloblasts containing an nulate lamellae or abnormally long strands of endoplasmic reticulum are often in active DNA synthesis As a high proportion of the ultrastructurally abnormal polychromatic megaloblasts belonged to the non-dividing late polychromatic cell compartment it was not surprising that most of the ultra structurally abnormal polychromatic megaloblasts were unlabelled with

Fig 1 Examples of ultrastructural abnormalities affecting the cytoplasm of some polychromatic megaloblasts. *a* Membrane-bound siderosome (above) and cir cular strand of rough endoplasmic reticulum (below) in a vitamin B_{12}-deficient megaloblast. ×50,000. *b* Several presumably autophagic vacuoles bounded by two concentric layers of membrane in a vitamin B_{12}-deficient megaloblast. The space between the two layers of membrane is more or less electron-lucent in the vacuoles marked 1 and 2 and is filled with electron-dense material in the vacuoles marked 3 and 4 ×25,000. *c* Electron microscope autoradiograph (developed with ID19) of an ^{3}H leucine-labelled, folate-deficient megaloblast containing a cir cular strand of endoplasmic reticulum One of the linear strands of endoplasmic reticulum in this cell profile is continuous with the nuclear membrane. ×30,000 *d* Electron microscope autoradiograph (developed with gold intensification and Elon ascorbic acid) of an H TdR-labelled vitamin B_{12}-deficient megaloblast showing two presumably autophagic vacuoles. The wall of the upper vacuole contains 2 concentric layers of membrane and that of the lower vacuole has 3 layers. The space between the layers has a cross-striated appearance. ×33,000.



1b
1d

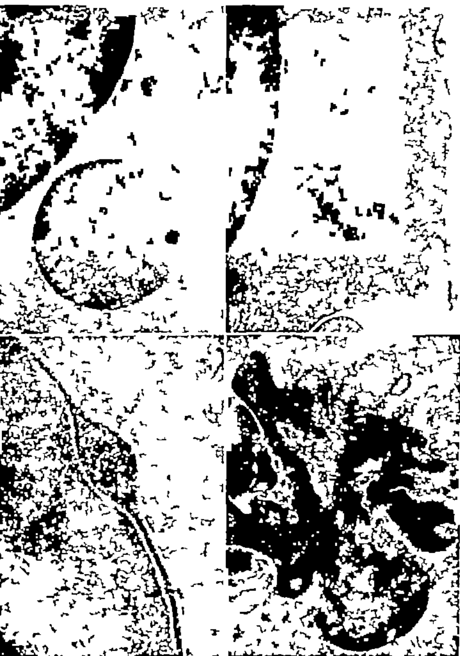
Table I Ultrastructural abnormalities encountered in some profiles of the polychromatic megaloblasts

Cytoplasmic abnormalities	Nuclear abnormalities
Long strands of endoplasmic reticulum (sometimes dilated or twisted into bizarre formations)	Reduced quantity of heterochromatin
Circular membranes enclosing portions of the cytoplasm, probably autophagic vacuoles (fig. 1 3a)	Membrane-bound nuclear clefts, usually within the heterochromatin (fig. 2c, d, 3c)
Annulate lamellae, mainly in the basophilic cells	Irregularly shaped or fragmenting nuclei (fig. 2d 3d)
Increased number of free ferritin molecules	Intra-nuclear inclusions (ferritin aggregates, mitochondria myelin figures, ? ribosomes) (fig. 3a b)
Very large siderosomes	Spongy appearance of heterochromatin
Mitochondrial degeneration (swelling, loss of cristae)	Absence of parts of the nuclear membrane (fig. 2d)
Iron-laden mitochondria	Myelinization of parts of the nuclear membrane
Inclusions consisting of nuclear material (Howell-Jolly bodies) (fig. 2a, b)	Separation of the nuclear membrane from the nucleus
Clustering of cytoplasmic organelles near the nucleus (fig. 4)	Dilation of the space between the 2 layers of the nuclear membrane (fig. 4)
Reduced electron density and ribosome content (fig. 4)	Heavy deposit of ribosomes on parts of the outer layer of the nuclear membrane

^{3}H TdR However a very few polychromatic megaloblasts containing presumably autophagic vacuoles (fig. 1d) irregularly shaped nuclei an incomplete nuclear membrane or membrane-bound nuclear clefts were found to be labelled with ^{3}H TdR.

Whereas in normal bone marrow virtually all of the erythroblast profiles were labelled with ^{3}H leucine, a small proportion of the erythroblast profiles in the vitamin B_{12} or folate-deficient bone marrows were either unlabelled or only very weakly labelled with this radioactive precursor These unlabelled or weakly labelled profiles usually showed ad-

Fig 2 Examples of ultrastructural abnormalities encountered in some vitamin B -deficient polychromatic megaloblasts. *a* Intracytoplasmic membrane bound inclusion consisting of both heterochromatin and euchromatin. ×23,000. *b* Intra-cytoplasmic inclusion resembling a chromosome fragment. ×45,000. *c* Membrane bound nuclear clefts within the heterochromatin of an otherwise normal-looking nucleus. ×45 000. *d* Grossly abnormal, irregularly shaped nucleus lacking part of its nuclear membrane and containing several intranuclear clefts. × 17,500



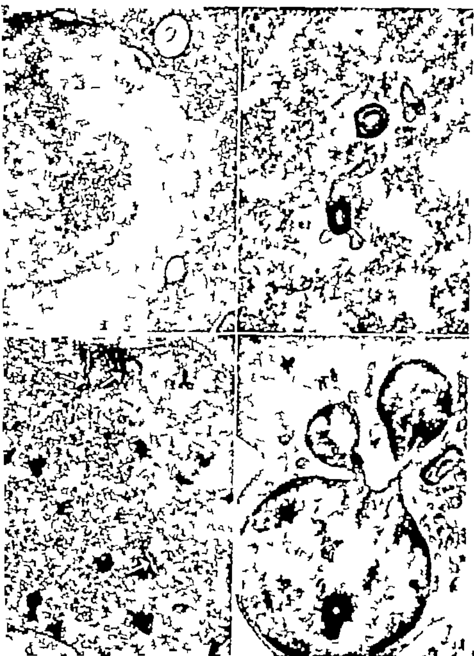
vanced degenerative changes, such as the dilation of the space between the two layers of the nuclear envelope, the clumping of cell organelles beside the nucleus, a scarcity of ribosomes, an abnormally electron lucent cytoplasm with a granular appearance, and the presence of several intracytoplasmic presumably autophagic vacuoles (fig. 4). Cells showing advanced degenerative changes were also either unlabelled or only very weakly labelled with ^{3}H uridine (fig. 4e). Several of the cell profiles showing less marked ultrastructural abnormalities including those containing abnormally long or circular strands of endoplasmic reticulum (fig. 1c), annulate lamellae, siderosomes, a few presumably autophagic vacuoles, iron laden mitochondria, irregular nuclei (fig. 3d), membrane-bound nuclear clefts (fig. 3c) or a combination of 2 or 3 of these abnormalities were found to be labelled with both ^{3}H-uridine and ^{3}H leucine. Some cell profiles containing an incomplete nuclear membrane were also labelled with both these radioactive precursors. Although many cells showing a distended nuclear envelope, paranuclear clumping of cytoplasmic organelles and a reduced electron density of the cytoplasm were unlabelled or only very weakly labelled with ^{3}H leucine (fig. 4), a few such cells showed a significant degree of labelling with this amino acid.

Most of the erythroblasts situated within the reticulum cells were either weakly labelled or unlabelled with ^{3}H leucine and ^{3}H uridine (fig. 4). However, a few of the phagocytosed erythroblasts were found to be more strongly labelled with ^{3}H leucine. The intensity of labelling over these labelled erythroblasts was higher than that in the cytoplasm of the reticulum cells containing them.

Discussion

This study has demonstrated a wide variety of ultrastructural abnormalities in the erythroblasts of vitamin B_{12} or folate-deficient patients.

Fig. 3. Nuclear abnormalities encountered in some vitamin B_{12}-deficient polychromatic megaloblasts. *a* Intranuclear inclusion consisting of an aggregate of electron-dense particles, presumably containing iron. This cell profile also includes an intracytoplasmic vacuole containing a myelin figure. ×22,500. *b* Intranuclear myelin figures. × 28,000. *c* Electron microscope autoradiograph of an ^{3}H-uridine-labelled megaloblast showing several intranuclear clefts (arrows). ×17,500. *d* Electron microscope autoradiograph of an ^{3}H leucine-labelled megaloblast containing an irregular nucleus. ×11,250.



However apart from the presence of an abnormally small quantity of ro hr matin in th nucleus, most of the other abnormalities affecting p l ch o natic erythroblasts (table I) were usually encountered in small proportion of these cell profiles. In particular only a few blasts howed advanced degenerative changes such as the disten) the pace between the 2 layers of the nuclear membrane, a r reau tion in the electron density of the cytoplasm the clumping o gan lles near the nucleus, the presence of multiple presumably a uoles and a scarcity of ribosomes These findings contrast he of GOODMAN *et al* [5] who studied 4 patients with vitamin) folate deficiency and found degenerative changes in erythroblasts ch had been phagocytosed within the reticulum cells but not in the non-phagocytosed erythroblasts.

Most of the intracytoplasmic inclusions consisting of nuclear material were completely surrounded by a double membrane which was indistinguishable from nuclear membrane (fig. 2a) Clearly the possibility that some of these membrane-bound inclusions were continuous with the main nuclear mass cannot be excluded without serial sectioning. Nevertheless, it seems likely that a proportion of these inclusions are not connected with the main nuclear mass and correspond to some of the Howell Jolly bodies seen under the light microscope An occasional Howell

Fig 4 *a* Two folate-deficient, non-phagocytosed erythroblasts (marked 1 and) illustrating several ultrastructural features of advanced degeneration. They contain a granular abnormally electron-lucent cytoplasm which is deficient in ribosomes. Their cytoplasmic organelles are clumped near the nucleus and the space between the two layers of their nuclear membrane is expanded. ×5,000 *b c* Electron microscope autoradiographs of folate-deficient marrow cells which had been incubated with ^{3}H leucine for 1 h. The erythroblast (marked 3) at the centre of *b* shows advanced degenerative changes, does not lie within a reticulum cell and is unlabelled with ^{3}H-leucine The surrounding cells had incorporated this amino acid. The erythroblast at the centre of *c* lies within an ^{3}H leucine-labelled reticulum cell but is itself virtually unlabelled with ^{3}H leucine. *b* ×4,500 *c* ×7,500 *d* An ^{3}H leucine-labelled, vitamin B_{12}-deficient erythroblast showing advanced degenerative changes. This cell is situated within a reticulum cell. ×5,000 *e f* Electron microscope autoradiographs of vitamin B -deficient marrow cells which had been incubated with ^{3}H uridine for 1 h. The cell marked 4 shows advanced degenerative changes, does not lie within a reticulum cell and is virtually unlabelled with ^{3}H-uridine The adjacent neutrophil myelocyte and erythroblast are both labelled with this precursor The erythroblast at the centre of *f* lies within an ^{3}H-uridine labelled reticulum cell but is itself unlabelled. *e* ×6,500 *f* ×4 400. *d f* The nucleus of the reticulum cell is marked N


1
2
3
N
N
4
N
N

Jolly body was not membrane bound consisted only of condensed chromatin and resembled a chromosome fragment (fig. 2b) Those Howell Jolly bodies which were surrounded by a nuclear membrane could have either arisen by karyorrhexis or by the organisation of a nuclear membrane around chromosome fragments which had become isolated outside the nucleus after the previous mitosis

None of the ultrastructural abnormalities listed in table I are specific for vitamin B_{12} or folate deficiency and most of them appear to represent non specific reactions to cellular injury Thus (1) various types of autophagic vacuoles have been described in the erythroblasts of patients with homozygous β thalassaemia [1 13 18 19] alcohol-induced sideroblastic anaemia [6] and congenital dyserythropoietic anaemia [11] and in human yolk sac erythroblasts [3] (2) large siderosomes, iron laden mitochondria and an increased number of free ferritin molecules within the cytoplasm have been described in primary acquired sideroblastic anaemia [1 10 13 16] secondary sideroblastic anaemia [4 6 13] hereditary sideroblastic anaemia [7 20] homozygous β-thalassaemia [1 14 19] the congenital dyserythropoietic anaemias [8 11 12] aplastic anaemia [2] and in erythroblasts from human yolk sacs [3] (3) various anomalies of the nuclear membrane have been reported in homozygous β-thalassaemia [14 19] the congenital dyserythropoietic anaemias [8 11 12] and aplastic anaemia [2] (4) membrane bound nuclear clefts and irregularly shaped or fragmenting nuclei have been described in erythroblasts from human yolk sacs [3] and patients with aplastic anaemia [2] (5) intra-nuclear ferritin aggregates have been described in homozygous β thalassaemia [14 19] and (6) intranuclear myelin figures have been reported in the congenital dyserythropoietic anaemias [8]

The data from the electron microscope autoradiographs indicate that siderosomes, iron laden mitochondria, a few autophagic vacuoles, irregularly shaped nuclei incomplete nuclear membranes or membrane-bound nuclear clefts may be present in cells synthesising DNA RNA and protein However all these biosynthetic processes were either arrested or markedly depressed in most of the erythroblasts showing advanced degenerative changes. Such grossly abnormal cells are probably eventually phagocytosed by the reticulum cells

Some of the phagocytosed erythroblasts were labelled with ^{3}H leucine indicating that a total arrest of protein synthesis does not always precede the phagocytosis of an erythroblast. The phagocytosed erythroblasts which were labelled with ^{3}H leucine were presumably engaged in

protein synthesis and labelled with this radioactive amino acid shortly prior to their phagocytosis. Two alternative explanations for the presence of ^{3}H leucine-labelled phagocytosed erythroblasts are (1) that protein synthesis can continue within a proportion of the phagocytosed erythroblasts soon after their phagocytosis and (2) that at least some of the radioactivity found within the phagocytosed erythroblasts is present in hydrolytic enzymes synthesised by the reticulum cells and subsequently transported into the phagocytosed cells, rather than in newly synthesised erythroblast protein

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Jolly body was not membrane bound consisted only of condensed chromatin and resembled a chromosome fragment (fig. 2b) Those Howell Jolly bodies which were surrounded by a nuclear membrane could have either arisen by karyorrhexis or by the organisation of a nuclear membrane around chromosome fragments which had become isolated outside the nucleus after the previous mitosis

None of the ultrastructural abnormalities listed in table I are specific for vitamin B_{12} or folate deficiency and most of them appear to represent non specific reactions to cellular injury Thus (1) various types of autophagic vacuoles have been described in the erythroblasts of patients with homozygous β thalassaemia [1 13 18 19] alcohol-induced sideroblastic anaemia [6] and congenital dyserythropoietic anaemia [11] and in human yolk sac erythroblasts [3] (2) large siderosomes iron laden mitochondria and an increased number of free ferritin molecules within the cytoplasm have been described in primary acquired sideroblastic anaemia [1 10 13 16] secondary sideroblastic anaemia [4 6 13] hereditary sideroblastic anaemia [7 20] homozygous β thalassaemia [1 14 19] the congenital dyserythropoietic anaemias [8 11 12] aplastic anaemia [2] and in erythroblasts from human yolk sacs [3] (3) various anomalies of the nuclear membrane have been reported in homozygous β-thalassaemia [14 19] the congenital dyserythropoietic anaemias [8 11 12] and aplastic anaemia [2] (4) membrane bound nuclear clefts and irregularly shaped or fragmenting nuclei have been described in erythroblasts from human yolk sacs [3] and patients with aplastic anaemia [2] (5) intra-nuclear ferritin aggregates have been described in homozygous β-thalassaemia [14 19] and (6) intranuclear myelin figures have been reported in the congenital dyserythropoietic anaemias [8]

The data from the electron microscope autoradiographs indicate that siderosomes, iron-laden mitochondria a few autophagic vacuoles irregularly shaped nuclei, incomplete nuclear membranes or membrane bound nuclear clefts may be present in cells synthesising DNA RNA and protein However all these biosynthetic processes were either arrested or markedly depressed in most of the erythroblasts showing advanced degenerative changes. Such grossly abnormal cells are probably eventually phagocytosed by the reticulum cells.

Some of the phagocytosed erythroblasts were labelled with ^{3}H leucine indicating that a total arrest of protein synthesis does not always precede the phagocytosis of an erythroblast. The phagocytosed erythroblasts which were labelled with ^{3}H leucine were presumably engaged in

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Idiopathic Acquired Refractory Sideroblastic Anemia
Banded Chromosome Analysis in Six Patients

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Key Words. Chromosome banding · Glutathione reductase · Hemochromatosis · Karyotype · Sideroblastic anemia

Abstract. Chromosome analyses with banding were performed on six patients with idiopathic acquired refractory sideroblastic anemia (IARSA). One patient was found to have an extra chromosome No. 8, but had a normal level of red cell glutathione reductase. Bone marrow chromosomes from the other patients showed a normal karyotype. 28 patients with IARSA, including our 6 patients, have had chromosomal analyses. T   consistent chromosomal abnormalities have been described +8 in three patients and 20q– in three others. Despite the presence of chromosomal abnormalities in about one half of the patients, no patient has yet developed acute myelogenous leukemia. Several have died of hemochromatosis. The presence of a chromosomal abnormality appears to hav no influence on the early course of IARSA.

Idiopathic acquired refractory sideroblastic anemia (IARSA) is a disease of the fifth and sixth decades. It is associated with a dimorphic population of red blood cells, a hypercellular bone marrow resulting from erythroid hyperplasia, hyperferremia, the presence of abnormal ('ringed') sideroblasts, and abnormalities in porphyrin metabolism [15, 18]. The clinical course of patients with IARSA is usually benign, with patients having a median survival of 10.0 years [18]. Controversy exists as to whether IARSA terminates in acute myeloblastic leukemia (AML) or re

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Table I Laboratory findings in six patients with IARSA

Patient	Age years	Sex	Hb g%	MCV µm	MCHC %	Reticulocytes %	WBC 10^3	Platelets 10^3	Serum Fe, µg%	Bone marrow cellularity %	M/E ratio	Ringed sideroblasts %
T B.	67	M	7.2	85	40	5.2	5.2	480	210	80	1/1	60
F E.	53	M	6 4	108	28	2.9	3.9	225	195	70	1/2	50
E. H.	71	F	8.3	96	35	2.7	9 4	260	165	80	1/5	70
F L.	61	M	7 4	100	30	3.9	3.9	433	155	70	1/1 5	50
N. L.	48	M	6.6	92	32	4.7	4.7	530	200	70	1/3	30
J T	56	M	9 8	102	29	4 4	6 4	390	180	90	1/3	40

Normal 60–120 µg%

as red-cell precursor with five or more iron-containing granules over one-third or more of the nuclear circumference. (4) Mild to moderate megaloblastosis on the bone marrow smear in the erythroid series, absence of any bi- or trinucleated ery throid precursors, and negative PAS cytochemistry of the marrow smear (5) Absence of any hematologic improvement with pyridoxine.

Cytogenetic analysis was performed at least once on all six patients, and sec ond sample was obtained from five. This included direct analysis of marrow specimens as well as 72-hour cultures of peripheral blood lymphocytes stimulated with PHA to reflect the patients constitutional chromosomal patterns.

The chromosomes were analyzed by use of standard Giemsa-stained slides and slides stained with quinacrine mustard for fluorescence, as previously described [25]. The chromosomes were identified according to the Paris nomenclature [19]; the kar yotypes are presented as recommended under this system

The observation of at least two 'pseudodiploid' or hyperdiploid cells, or of three hypodiploid cells showing the same bnormality was considered evidence for the presence of an abnormal clone patients with such clones were classified as abnor mal. Patients whose cells showed no alterations, or in whom the alterations involved different chromosomes in different cells, ere considered to be normal

Red-cell enzyme determinations were performed on 15 ml of venous blood, obtained in ACD vacutainer® tubes and refrigerated. The quantitative analyses for glutathione reductase, pyruvate kinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconat dehydrogenase were performed by Dr Henri Frischer by method previously described [10–12]. The activities of the following erythrocytic enzymes were also tested: adenylat kinase, phosphofructokinase, fructoaldolase, glyceraldehyde-3-phosphat dehydrogenase, and phosphoglycerate isomerase.

mains a chronic refractory anemia associated with a long survival [5 9] KUSHNER *et al* [18] in a review of IARSA found only 3 of 61 patients who died of AML [18] Two of the three died after 7 and 13 years, respectively the duration of the disease was not given for the last patient

Chromosomal changes in IARSA were first reported in 1966 [6 21] and have been confirmed subsequently [1 4 13 14 16 17] The changes most frequently observed have been the addition of a C group chromosome [1 14 16 21] or a deletion or pericentric inversion of one F chromosome [4 6] The chromosomal changes are an acquired somatic mutation affecting the marrow cells, since phytohemagglutinin (PHA)-stimulated lymphocytes and cultured fibroblasts have a normal karyotype. Whether these mutations are evidence of malignancy or predispose to the malignant transformation of cells is unknown. The deficiency of many of these chromosomal studies is that few were done by the new banding techniques with either quinacrine mustard or Giemsa [3 25] These banding techniques permit the precise identification of each human chromosome and the detection of structural rearrangements that do not alter the shape of the chromosome (i e balanced translocations) They also allow the precise identification of deleted or extra chromosomes.

The purpose in this study was to determine the frequency and type of chromosomal abnormalities in IARSA and to attempt to correlate the chromosomal changes with possible associated biochemical markers In particular because of a previous report [8] that an increased level of glutathione reductase was observed in patients with an additional No 8 an analysis of red cell enzymes was carried out on the one patient in this series who showed an abnormal karyotype

Materials and Methods

All six patients with a diagnosis of IARSA from whom material for chromosomal analysis was available from January 1, 1971 to December 31 1974 were included in the study The clinical and laboratory course, including iron studies, peripheral smears, bone marrow smears, and marrow sections, was reviewed by two of us (J B and H. M G)

The following criteria were used to establish the diagnosis of IARSA [18] (1) The presence of anemia not associated with an illness or drug or toxin exposure (including alcohol), and the presence of a dimorphic population of red blood cells on peripheral smears. (2) Hyperferremia, with normal vitamin B , and folate levels. (3) Hyperplastic bone marrow with erythroid hyperplasia, and 'ringed sideroblasts on the Prussian blue iron stain of bone marrow sections. A ringed sideroblast is defined

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Red-cell enzyme determinations were performed on 15 ml of venous blood, obtained in ACD vacutainer® tubes and refrigerated. The quantitative analyses for glutathione reductase, pyruvate kinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase were performed by Dr Henri Frischer by method previously described [10–12]. The activities of the following erythrocytic enzymes were also tested, adenylate kinase, phosphofructokinase, fructoaldolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate isomerase.

Table II Cytogenic analysis of bone marrow (BM) or peripheral blood (PB) cells in six patients with IARSA

Patient	Date	Source	Number of cells[a]						Karyotype
			number of chromosomes				other chromosomal abnormalities	total	
			44	45	46	47			
T B	3–6–74	BM	1		8 (5)[b]	14 (12)	1 (1)[c]	[illegible]6 (18)	47 XY +8
	4–26–74	PB ([illegible]4 h)			4 (0)				46, XY
		PB (72 h)	3 (2)	3 (3)	19 (14)			[illegible]5 (19)	46, XY
F E.	10–25–73	BM			41		2[d]	43	46, XY
	1–15–74	BM		3 (3)	15 (6)		[illegible] (1)[e]	20 (10)	46, XY
E. H.	2–8–72	BM		2	45			47	46, XX
	7 19–75	BM	4 (1)	3 (3)	25 (9)			45 (13)	46, XX
F L.	5–21–71	BM	1	6	30		3	40	46, XY
	5–[illegible]6–73	BM	3 (0)	5 (1)	47 (4)	1 (1)[f]		56	46, XY
N. L.	10– 4–74	BM	5 (2)	4 (2)	18 (7)			27 (6)	46, XY
J T	6–6–75	BM		3 (2)	19 (10)[g]	1 (1)[h]		23 (13)	46, XY
	6–6–75	PB (72 h)	1	4 (3)	6 (4)			11 (7)	46 XY
	10–10–75	BM	8 (4)	5 (4)	13 (6)		1	27 (15)	46, XY

Number in parentheses indicates number of cells examined with quinacrine fluorescence.

[b] Only two of these were normal 3 had +8 minus some other chromosomes.
Cell had 48 +8 plus abnormal 9?

[d] Broken tetraploids with 53 and 58 chromosomes.
Tetraploid (92 chromosomes).

[f] Cell had an extra C which could be a No. 8.
One cell had an unidentified structural rearrangement.

[h] One cell had a large submetacentric marker chromosome.

Results

Data on the six patients are summarized in table I All patients had a hypercellular bone marrow with ringed sideroblasts present in marrow sections and all had mild to moderate erythroid megaloblastosis

The results of chromosomal analyses are presented in table II Five of the patients had a modal chromosome number of 46 without any dele

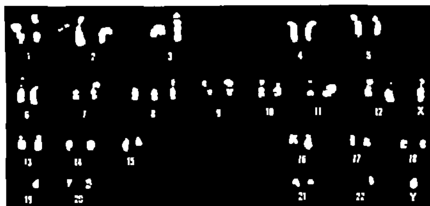


Fig 1 Karyotype of bone marrow cell obtained from patient (T B.) with IARSA and stained with quinacrine mustard. An additional chromosome No. 8 is the only abnormality detected, the karyotype of this cell is 47 XY +8.

Table III. Red cell enzyme assays in the patient with trisomy 8 (T B.)

Enzyme	Patient	Normal values		
		mean	range	number of determinations
Glutathione reductase	6.7 6.9	8.9	6.0–12.4	114
Pyruvate kinase	8.7	11.7	6.1 15.7	50
Glucose-6-phosphate dehydrogenase	15.7	9.4	6.1 13.7	114
6-Phosphogluconate dehydrogenase	10.4	8.3	6.0–12.12	114

Performed by Dr Hervu F [illegible], Rush-Presbyterian St. Luke Medical Center all *enzymes are expressed as μmoles of* NADP(H) *oxidized (reduced)* min^{-1} g^{-1} *Hb* (37 °C). The following erythrocytic enzyme activities were normal adenylate kinase, phosphofructokinase, fructoaldolase, glyceraldehyde-3-phosphate dehydrogenase and, phosphoglycerate kinase.

tions, translocations, or inversions. One patient (T B.) had 47 chromosomes, with an extra No. 8 that has persisted until the present time (fig 1). Two other patients had inconsistent abnormalities. Patient F L. had one cell with 47 chromosomes and an extra C. This chromosome could not be identified with certainty although it could have been an extra No. 8 The second patient (J T) had two cells with two different

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	7-19-75	BM	4 (1)	3 (3)	25 (9)			45 (13)	46, XX
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	5-26-73	BM	3 (0)	5 (1)	47 (4)	1 (1)[f]		56	46, XY
N L.	10- 4-74	BM	5 (?)	4 (2)	18 (2)			[illegible]7 (6)	46, XY
J T	6-6-75	BM		3 (?)	19 (10)[g]	1 (1)[h]		23 (13)	46, XY
	6-6-75	PB (72 h)	1	4 (3)	6 (4)			11 (7)	46, XY
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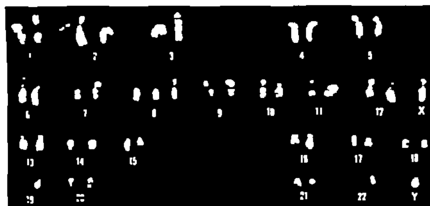


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Performed by Dr Henri Frischer, Rush-Presbyterian St. Luke Medical Center all enzymes are expressed as μmoles of NADP(H) oxidized (reduced) min g Hb (37°C). The following erythrocytic enzyme activities were normal adenylate kinase phosphofructokinase, fructoaldolase, glyceraldehyde-3-phosphate dehydrogenase and, phosphoglycerate kinase.

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E. H.	2-8-72	BM		2	45			47	46, XX
	7-19-75	BM	4 (1)	3 (3)	25 (9)			45 (13)	46, XX
F. L.	5-1-71	BM	1	6	30		3	40	46, XY
	5-26-73	BM	3 (0)	5 (1)	47 (4)	1 (1)[f]		56	46, XY
N. L.	10-24-74	BM	5 (?)	4 (2)	18 (2)			27 (6)	46, XY
J. T.	6-6-75	BM		3 (2)	19 (10)[g]	1 (1)[h]		23 (13)	46, XY
	6-6-75	PB (72 h)	1	4 (3)	6 (4)			11 (7)	46, XY
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had IARSA according to the criteria defined by KUSHNER [18] In five reported patients with sideroblastic anemia, associated illnesses, drug exposure, or malignancy were present [13] one patient had chronic erythremic myelosis [2] one had a congenital sideroblastic anemia [7] and one had a pyridoxine-responsive sideroblastic anemia [13] For two others, either no chromosomal data were available for analysis or the analysis had been performed only when overt leukemia developed [10 24] With the addition of our 6 patients, there was a total of 28 reported cases of IARSA on whom chromosomal analyses [1 4 6, 13 14 16 17 21] (table IV) were performed early in the course of the disease.

The presence of an extra C group chromosome has been confirmed in 5 of these 28 cases of IARSA [1 14 16, 21] In three instances where banding techniques were used, including the six cases in the present study the extra C group chromosome has been a No. 8 [14 16] The presence of extra chromosome No 8 in bone marrow cells has now been described in IARSA, other refractory anemias, myelofibrosis, acute leukemia, and chronic myelogenous leukemia [22, 23]

Three patients with IARSA have been described who had a deletion of a portion of one F group chromosome [4 6] COHEN *et al* [4] reported on a patient with IARSA who had a deletion of the long arm of chromosome No. 20 (20q–) identified by fluorescent banding techniques. Two of 6 patients with IARSA reported by DE GROUCHY *et al.* [6] had a deletion of an F chromosome, and three had what appeared to be a pericentric inversion of one of the F group chromosomes. The deleted F in the former patients was a No 20 [DE GROUCHY personal commun.]

Two patients, males aged 59 and 68 years, showed a loss of the Y chromosome [13 17] The significance of this observation is uncertain since 10 to 20% of hematologically normal males in this age group would be lacking a Y chromosome in their bone marrow cells [20] Another patient had a possible translocation to the long arm of one No. 2 chromosome [13] Fourteen patients had no detectable chromosomal abnormality

DE LA CHAPELLE [8] reported that 3 of 4 patients with an extra No. 8 had an increased level of erythrocyte glutathione reductase (GSSG-R). Two of these patients had IARSA the GSSG-R was elevated in one and normal in the other One patient of the present series had an extra No 8 and a normal level of GSSG-R as well as of the other red cell enzymes studied except for an elevated level of G-6-PD Analysis of further patients will be needed for clarification of the relationship between an extra No 8 and the level of GSSG-R.

Table II Cytogenetic analysis on patients with IARSA

Patients	Age/sex	Chromo-somes	Abnormality	Reference
Case 2	62/M	47	+C	21
Observation 1	70/M	46	Fq−	6
Observation 2	67/M	46	none	6
Observation 3	54/M	46	Fq−	6
Observation 4	68/M	46	Inv(F)	6
Observation 5	63/M	46	inv(F)	6
Observation 6	77/M	46	inv(F)	6
Case 1	68/M	45	−Y or −G	13
Case 2	89/F	46	none	13
Case 4	86/F	46	2q+	13
Case 5	84/M	46	none	13
	51/M	47	+8[b]	14
	50/M	47	+C	1
	38/M	46	20q−[b]	4
Case	69/F	47	+8	16
3 patients	N G./F	46	none	17
3 patients	N G /M	46	none	17
1 patient	59/M	45	−Y[b]	17
T B.	67/M	47	+8[b]	present study
F E.	53/M	46	none	present study
E. H.	71/F	46	none	present study
F L.	61/M	46	none	present study
N L.	48/M	46	none	present study
J T	56/M	46	none	present study

N G = Not given
Inv(F) is a pericentric inversion of one F chromosome.
Banding techniques used.

structural rearrangements. The level of red-cell enzymes was analyzed in the one patient (T B) whose bone marrow cells showed a +8 karyotype The sample was obtained 7 weeks after a transfusion of 5 units of packed red blood cells. The results are presented in table III.

Discussion

Although chromosomal analyses have been performed in 32 patients with sideroblastic anemia [1 2, 4 6, 7 13 14 16 17 21 24] only 22

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The presence of an extra chromosome No 8 does not appear to influence survival in IARSA also no patient with IARSA and an extra No 8 has been reported who developed acute leukemia. Most patients with IARSA have died from hemochromatosis a complication of iron overload. The median survival for the six patients reported here is 24+ months the patient with the +8 abnormality is alive and well at 30 months, with no evidence of leukemia Longer follow up is needed to provide information as to whether the presence of an extra chromosome No 8 predisposes to a leukemic outcome however current evidence suggests that this abnormality does not influence the initial course of IARSA

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dicates a disturbance of heme and/or hemoglobin synthesis [1 2, 8 9–11]

It was the aim of this study to compare various techniques proposed for iron staining of bone marrow and to establish criteria for semiquantitative and qualitative evaluation of sideroblasts. We propose the introduction of a *sideroblast score instead of the customary percentage of iron-*containing erythroblasts. Our preliminary results suggest that the sideroblast score provides valuable information concerning the availability of transport iron in relation to hemoglobin synthesis and erythroid proliferation.

Material and Methods

Bone marrow specimens were obtained from iliac crest, occasionally from sternum or in connection with bone marrow biopsy Aspirates were immediately suspended in 1 / EDTA solution. Particles were then carefully smeared onto chemically clean glass slides and left to dry under cover from external contamination.

Iron staining Until now the procedure of LÜDIN [12] was routinely used in our laboratory The suitability of this method concerning the ease of performance, detectability of storage iron and sideroblasts was compared with procedures described by WILLIAMS *et al.* [13], HANSEN and WEINFELD [2], MCFADZEAN and DAVIS (quoted by KAPLAN [7]) and BILGER and TETZNER [14].

This latter method has been modified by HECKNER [15] and includes the following steps: (1) smears should be completely dry (air); (2) fixation with pure methanol (5 min), drying (air) (3) staining in acid potassium ferrocy nide (5 min). Acid solution should be prepared immediately prior to staining by adding 0.5 ml of 37% HCl to 50 ml of 1 % potassium ferrocyanide, and (4) counterstaining with Mayer's hemalum. 1 g hemalum is dissolved in 1,000 ml distilled water Subsequently 0.2 g $NaIO_3$, 50 g potassium alum, 50 g chloralhydrate and 1 g citric acid are added the solution is immediately ready for use and stable. W found that additional short flushing with distilled water improved the stability of staining [16] and removed artifacts.

Sideroblast score A numerical value is being attributed to each of 100 mature erythroblasts Normoblasts are clearly recognized by their dense nuclei. (In megaloblastic or dyserythropoetic anemias it is more difficult to differentiate maturation stages.) The following scores were arbitrarily attributed to each normoblast or to abnormal iron-containing erythroid precursor: 0 = no stainable iron; 1 = single, *fine* iron granule; 2 = two or more *fine* granules, 3 = one or more (<5) *coarse* granule( ), and 4 = numerous (>5) *coarse* granules.

Storage iron A scale similar to the one proposed by RATH and FINCH [17] was used. The following six stages were independently differentiated by tw persons: 0 = no storage iron, 1/6 = traces of storage, iron detectable; 2/6 = more diffuse, but weak staining; 3/6 = low normal 4/6 = normal, 5/6 = moderate increase, and 6/6 = massive hemosiderosis. In marrow with low iron content, staining artifacts or external contamination, g with dust particles, should be recognized.

Acta haemat. 57 24–31 (1977)

Sideroblast Score A Sensitive Indicator of Iron Deficiency and Hypoproliferative Anemia

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Key Words Anemia Bone marrow iron Iron metabolism Iron staining Sideroblasts

Abstract The detection and enumeration of sideroblasts depend critically on the method used for iron staining of bone marrow smears. Several methods proposed for semiquantitative evaluation of bone marrow hemosiderin (iron stores) were compared with respect to their suitability for detection of normal and abnormal sideroblasts. Instead of the customary percentage of sideroblasts, the introduction of a sideroblast score is proposed and its diagnostic relevance was prospectively studied. Low sideroblast scores are associated with iron deficiency and hypoproliferative anemia. A normal sideroblast score, despite the absence of stainable hemosiderin, excludes the diagnosis of severe iron depletion. Elevated sideroblast scores may be correlated either with iron overload and/or sideroblastic (sideroachrestic) anemias.

Histochemical methods are suitable for detection of mainly two types of nonheme iron in bone marrow (1) storage iron (hemosiderin) predominantly within cells of the reticuloendothelial system and (2) granular material (probably ferritin) in the cytoplasm of erythroblasts (sideroblasts) or red cells (siderocytes) The clinical importance of histochemical evaluation of storage iron is well established [1–5] However available methods seem not to be uniformly suitable for detection of sideroblasts. Thus, controversy still exists concerning the proportion of erythroid precursors containing stainable iron under normal conditions [1 2 6–8] Without this basic knowledge it is difficult to interprete disorders associated with low or increased sideroblast counts. Most authors, however agree that the presence of coarsely granulated or ringed' sideroblasts in

Table I. Correlation of sideroblast score with iron kinetics

Clinical diagnosis	Sidero-blast score	Serum iron µg 100 ml	Trans-ferrin, satura-tion,	Plasma clear ance of iron min	Erythroid iron turnover	Iron store in bone marrow
Chronic rheumatoid arthritis	3	59	31.7	36	normal	3/6
Hodgkin's disease (II A)	6	19	11.5	31	normal	3/6
Hodgkin's disease (IV B)	42	14	6.8	25	normal	
Hodgkin's disease (I A)	53	61	20.8	75	normal	3/6
Sideroblastic anemia	36	94	48.2	43	increased	3/6
Aplastic anemia	88	70	26.4	163	decreased	2/6
Anemia, megaloblastoid erythropoiesis	94	81	40.7	48	increased	4/6
Felty's syndrome (hyper cellular marrow)	97	77	12.5	27	increased	4/6
Hematologically normal control	91	56	24.9	76	normal	5/6

ready suggest that no simple correlation between the sideroblast score and any single other laboratory result can be regularly expected. Figure 1 illustrates the correlation of the sideroblast score with semiquantitatively evaluated iron stores. Because of the overall poor correlation shown in this figure it is of interest to give additional information on individual patients (table II). Thus, five patients with hypoproliferative anemia had a markedly decreased sideroblast score, despite adequate iron stores. The main clinical diagnoses were chronic rheumatoid arthritis, malignant lymphoma and chronic infection (patient No. 1 4 7 8 and 11). Table II also indicates that the sideroblast score may be of interest in specimens where storage iron is not detectable because of inadequate cellularity (patient No. 5 and 19 not shown in figure 1).

The shaded area on the figure includes a grossly estimated normal range, including borderline abnormalities of iron stores (2/5 and 5/5). HEILMEYER [15] indicates a normal range of 30–60% of sideroblasts containing fine granules. Assuming that 30% of sideroblasts contain a single

Results

In a first retrospective study 100 bone marrows examined sequentially in 1974 were reevaluated Iron staining according to the method of LÜDIN [12] had been performed on 88 smears, whereas the remaining bone marrows originated from patients with leukemia, etc. where evaluation of iron stores was not of primary interest. According to a previous study this simple procedure was suitable for evaluation of iron stores and detection of sideroblastic anemia [8] but less sensitive for recognition of normal sideroblasts. The present evaluation produced some surprising results concerning iron stores iron staining had only been specifically requested on eight occasions, twice with the question on possible increase, six times concerning decrease of iron stores. By routine iron staining we found however 34 marrows with no detectable storage iron and an additional seven marrows with decreased stainable iron Iron stores were markedly increased in 16 marrows. Abnormal sideroblasts were found in four instances In conclusion abnormal iron stores were suspected in more than half of this particular group of patients.

Comparison of different methods, performed on smears originating from the same patient indicated that all other procedures were equally suitable for detection of iron stores. However they varied mainly with respect to clear-cut staining of iron granules in normoblasts of those specimens where neither iron deficiency nor other disturbances of iron metabolism were to be expected. The methods proposed by WILLIAMS *et al* [13] and HECKNER [15] were both sensitive for detection of normal sideroblasts The latter method was finally preferred because it permits easier recognition of morphological features of nonerythroid cells and staging of erythropoiesis.

An additional 17 marrows were then simultaneously stained by the methods of LÜDIN [12] and HECKNER [15] These included specimens from two patients with sideroblastic anemia The method of LÜDIN again did not permit recognition of normal sideroblasts, but clearly revealed abnormal sideroblasts. Staining according to HECKNER showed normal sideroblasts in 16 preparations Abnormal sideroblasts were characterized by their coarse granulation (using both methods)

In subsequent prospective studies the modified procedure of HECKNER [15] was used exclusively and the sideroblast score was compared with other parameters A small number of patients, on whom iron kinetics were studied, was thus evaluated The results summarized in table I al

Table II Comparison of clinical diagnosis, sideroblast score and iron store

Patient No.	Clinical diagnosis	Sideroblast score	Iron store
1	Chronic rheumatoid arthritis	3	3/6
2	Felty's syndrome	97	4/6
3	Bronchial carcinoma	91	5/6
4	Hodgkin's disease (II A)	6	3/6
5	Hodgkin disease (IV B)	42	?
6	Hodgkin's disease (I A)	53	3/6
7	Malignant lymphoma	9	4/6
8	Malignant lymphoma	4	4/6
9	Idiopathic pancytopenia, sideroblastic anemia	236	5/6
10	Pernicious anemia	94	4/6
11	Pernicious anemia	198	4/6
12	Pernicious anemia, remission	53	4/6
13	Aplastic anemia	88	2/6
14	Immunogenic thrombocytopenia	108	5/6
15	Polycythemia vera	7	0/6
16	Acute myelogenous leukemia	67	3/6
17	Lymphadenopathy viral?	228	6/6
18	Chronic alcoholism	171	5/6
19	Anemia, etiology undetermined	124	?
20	Anemia, etiology undetermined	25	5/6
21	Anemia, chronic infection	2	4/6
22	Iron deficiency anemia	3	0/6
23	Addison's disease	89	3/6
24	? bone marrow hemosiderosis	144	6/6

tainly not representative for a larger population [8]. Comparative analysis of several published procedures for iron staining [2, 7 12–15] indicates that all are equally suitable for evaluation of iron deposits within reticuloendothelial cells.

Minor technical differences seem to be more crucial for detection of iron-containing particles in the cytoplasma of normal sideroblasts by light microscopy The procedure described by Heckner [15] was found to be a particularly sensitive and simple method for this purpose. Introduction of a sideroblast score, rather than indiscriminate counting of sideroblasts (percentage), may be practically important in several respects: a normal sideroblast score, in the absence of sufficient bone marrow ('stroma') particles for evaluation of iron stores, excludes the diagnosis of severe iron

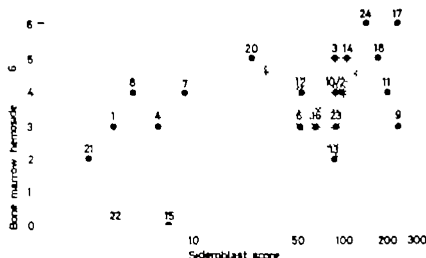


Fig 1 Correlation between semiquantitatively evaluated iron stores (bone marrow hemosiderin) and corresponding sideroblast scores. The shaded area indicates an approximate normal range.

iron granule (stage 1) the lowest normal sideroblast score would be 30 Since the presence of coarsely granulated sideroblasts in normal bone marrows is exceptional [8] the upper limit was defined as 60% of erythroblasts containing more than one fine granule (stage 2) with a corresponding score of 120 The group with a supposedly increased sideroblast score includes three patients with iron stores graded 5/5 or 6/6 (No 17 18 and 24) as well as a patient with untreated pernicious anemia (No 11) and, the highest score, a patient with refractory sideroblastic anemia (No 9) A very low sideroblast score was found as expected in iron deficiency anemia (No 22) and polycythemia vera (No 15)

Discussion

According to previous studies there is ample evidence suggesting that iron staining of bone marrow provides at least qualitative information on body iron stores [1–5 17] Our retrospective study on 88 specimens shows that it is indeed sensible to perform iron staining routinely in addition to Wright or Pappenheim staining for overall morphological evaluation However the high incidence of increased iron stores was rather surprising and the proportion of marrows with abnormal sideroblasts is cer

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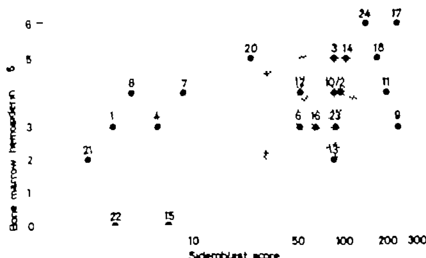


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deficiency The data presented in figure 1 and table II furthermore support the concept that the number of sideroblasts is reduced in hypoproliferative anemia e.g. in patients with tumor chronic infection rheumatoid arthritis etc. [17–19] The described method furthermore provides at least qualitative information on the availability and/or utilization of transport iron which might otherwise be obtained only by more elaborate techniques, such as iron kinetics or quantitative measurement of serum ferritin [20] Our preliminary data do not support the concept of BAINTON and FINCH [1] that there exists a close correlation between the number of sideroblasts and iron saturation of transferrin but are in agreement with the studies of HANSEN and WEINFELD [2] Evaluation of the sideroblast score in a few patients with increased bone marrow hemosiderin however indicates that an increased proportion of normal sideroblasts indeed reflects iron overload, unless erythroid proliferation and/or iron transport are suppressed Extremely high sideroblast scores are to be expected in sideroblastic anemia with iron overload The presence of coarse iron granules should however always be described as an abnormal feature of erythropoiesis, irrespective of the sideroblast score

In conclusion we propose that sideroblasts in bone marrow should receive more careful attention in analogy to the misused reticulocyte [21] Further studies are necessary to clearly establish normal values and to define the diagnostic relevance of the sideroblast score in various disorders of hematopoiesis

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Acta haemat. 57 3 –36 (1977)

Quantitation of Hb A_2 with DE-52 Microchromatography in Whole Blood as Screening Test for β-Thalassemia Heterozygotes[1]

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Key Words. Hb A_2 Hemoglobinopathies Microchromatography Thalassemia screening β Thalassemia

Abstract Hb A_2 was assayed by means of DE 52 microchromatography in hemolysates from 285 normal subjects and 223 β thalassemia heterozygotes. No overlap was found between both groups. Comparable results were observed analyzing whole blood samples collected in capillary tubes from 550 normal subjects and 295 β thalassemia heterozygotes. Our results demonstrate that this technique is useful in a screening program for β-thalassemia trait.

The increase of hemoglobin A_2 (Hb A_2) is the main diagnostic feature of β thalassemia carriers [1] Therefore a rapid and reproducible quantitation of Hb A_2 is a fundamental step in a screening program for this trait. A satisfactory estimation of Hb A_2 may be achieved by several methods. The most reproducible results have been obtained by starch-block electrophoresis [2] DEAE Sephadex or DEAE-cellulose chromatography [3–5] polyacrylamide gel electrophoresis [6] and cellulose-acetate electrophoresis with quantitation of Hb A_2 after elution of the hemoglobin fractions [1] A very simple and rapid microchromatographic method applicable to hemolysate, whole blood collected by fingerstick either in microhematocrit heparinized capillary tubes or on filter paper was recently introduced by Efremov *et al* [7] and successively modified by Huisman *et al* [8] The purpose of this paper is to present the results obtained using the modified method in the identification of thalassemia carriers in Sardinia.

[1] This work was supported by a research grant from Assessorato Igiene & Sanità della Regione Sarda

Table I Average values, standard deviations (SD) and ranges of Hb A_2 levels (%) with DE-52 microchromatography of hemoglobin solutions from hemolysates

	Number of cases	Mean	SD	Range
A	285	2.49	0.35	1.72–3.21
B	103	5.55	0.55	4.52–6.81
C	110	5.35	0.53	4.34–6.61

A = Normal subjects B = parents of children with homozygous β-thalassemia C = β-thalassemia heterozygotes.

Table II Average values, standard deviations (SD) and ranges of Hb A_2 levels (%) with DE 52 microchromatography of hemoglobin solutions from whole blood

	Number of cases	Mean	SD	Range
A	550	2.50	0.43	1.71–3.20
B	95	5.33	0.46	4.40–6.56
C	200	5.35	0.55	4.23–6.80

A = Normal subjects B = parents of children with homozygous β-thalassemia C = β-thalassemia heterozygotes.

Materials and Methods

Samples were obtained from parents of children with homozygous β-thalassemia and from individuals who participated in a screening program for β-thalassemia in Sardinia.

Blood was collected by venipuncture with heparin as anticoagulant or by finger stick in microhematocrit heparinized capillary tubes.

Microchromatography has been carried out according to Huisman *et al.* [8] using DE-52 microgranular (manufactured by Whatman Biochemicals Ltd.) in 0.5×5 cm chromatographic columns. The optical density of the eluate was determined at 415 nm (Spectrophotometer PM4, Carl Zeiss).

Results

Table I presents the results obtained with hemoglobin solutions from hemolysates of 103 parents of children with homozygous β-thalassemia,

Acta haemat. 57 3 -36 (1977)

Quantitation of Hb A_2 with DE 52 Microchromatography in Whole Blood as Screening Test for β-Thalassemia Heterozygotes[1]

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Key Words Hb A_2 Hemoglobinopathies Microchromatography Thalassemia screening β-Thalassemia

Abstract Hb A was assayed by means of DE 52 microchromatography in hemolysates from 285 normal subjects and 223 β-thalassemia heterozygotes. No overlap was found between both groups. Comparable results were observed analyzing whole blood samples collected in capillary tubes from 550 normal subjects and 295 β thalassemia heterozygotes. Our results demonstrate that this technique is useful in a screening program for β-thalassemia trait.

The increase of hemoglobin A_2 (Hb A_2) is the main diagnostic feature of β-thalassemia carriers [1] Therefore, a rapid and reproducible quantitation of Hb A_2 is a fundamental step in a screening program for this trait. A satisfactory estimation of Hb A_2 may be achieved by several methods The most reproducible results have been obtained by starch-block electrophoresis [2] DEAE Sephadex or DEAE-cellulose chromatography [3–5] polyacrylamide gel electrophoresis [6] and cellulose acetate electrophoresis with quantitation of Hb A_2 after elution of the hemoglobin fractions [1] A very simple and rapid microchromatographic method applicable to hemolysate, whole blood collected by fingerstick either in microhematocrit heparinized capillary tubes or on filter paper was recently introduced by Efremov *et al.* [7] and successively modified by Huisman *et al* [8] The purpose of this paper is to present the results obtained using the modified method in the identification of thalassemia carriers in Sardinia.

[1] This work was supported by a research grant from Assessorato Igiene & Sanità della Regione Sarda

Table III. Reproducibility of DE 52 microchromatography Hb A_2 values in %

	Material	Number of determinations	Mean	SD	Range
A	hemolysate	5	2.40	0.23	2.06–2.60
	whole blood	5	2.58	0.37	2.28–3.14
B	hemolysate	5	5.32	0.13	5.20–5.51
	whole blood	5	5.38	0.42	4.77–5.81
B	hemolysate	5	5.76	0.19	5.58–6.08
	whole blood	5	5.86	0.22	5.56–6.17
C	hemolysate	5	6.13	0.08	6.00–6.21
	whole blood	5	5.88	0.27	5.57–6.21
C	hemolysate	5	5.33	0.16	5.19–5.62
	whole blood	5	5.02	0.22	4.69–5.25

A = Normal subjects B = parents of children with homozygous β-thalassemia C = β-thalassemia heterozygotes.

Discussion

The DE-52 microchromatography is a simple and reliable method for the quantitation of Hb A_2. The main advantages of the method are the use of very small amounts of whole blood, the easy and rapid execution, the high reproducibility and the low costs. In addition, a high number of samples can be analyzed simultaneously. Presently in our laboratory a technician can determine the Hb A_2 levels in 50 samples of whole blood within 1 day. Another advantage of the method is the possibility of detecting minor changes in Hb A_2 levels.

It is important to emphasize that we have not encountered overlap between values from β-thalassemia heterozygotes and controls. Moreover the gap between the two groups was found to be large. In our experience the differentiation between β-thalassemia heterozygotes and controls was less definite when the Hb A_2 levels were determined by cellulose acetate electrophoresis. Microchromatography of whole blood collected in microhematocrit heparinized capillary tubes showed results similar to those obtained using hemolysates.

This findings demonstrate that this technique can be used in a screening program for β-thalassemia trait.

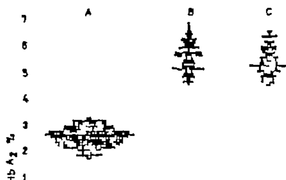


Fig 1 Hb A_2 levels (%) with DE 52 microchromatography of hemoglobin solutions from hemolysates. A = Normal subjects, B = parents of children with homozygous β-thalassemia C = β-thalassemia heterozygotes.



Fig 2 Hb A_2 levels (%) with DE 52 microchromatography of hemoglobin solutions from whole blood. A = Normal subjects B = parents of children with homozygous β thalassemia, C = β-thalassemia heterozygotes.

110 previously diagnosed β-thalassemia heterozygotes and 285 normal adults. There is no overlap between heterozygotes and controls. The values of Hb A_2 from heterozygotes identified in the screening program are nearly identical to those from known heterozygotes emphasizing the accuracy of the assay

Similar results were obtained with hemoglobin solutions from whole blood (table II). The reproducibility of the method is shown in table III

The level of Hb A_2 was significantly reduced in a child with Hb H disease (0 64%) and in one of his parents (0 60%).

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Inherited Erythrocyte Pyruvate Kinase Deficiency. Studies on 15 Members of Two Related Families[1]

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Key Words. Erythrocyte enzymes · Haemolytic anaemia · Pyruvat kinase deficiency · Red cell metabolism

Abstract. The case of a haemolytic non-spherocytic anaemia with pyruvate kinase (PK) deficiency is reported. The investigation concerns two families with low level of PK. In the propositus and in the members of his family we have also examined the behaviour of some enzymes and the concentration of red cell metabolites. We confirm the heterogeneity of the manifestation and agree that anaemias due to PK deficiency are complex forms in which the enzyme defect is only one of the symptoms.

The present work is a report of research carried out first on the erythrocytes of a subject with haemolytic anaemia accompanied by a low level of PK and also on the RBC of 13 of his relatives. The defect was evident on both the paternal and maternal sides of the family. In the propositus and in other subjects, we examined the activity level and other features of PK, and also the behaviour of enzymes and the concentration of red cell metabolites. These observations confirmed the remarkable heterogeneity of this kind of enzymopathy.

[1] The present work has been carried out with CNR funds.

The following abbreviations have been used: G6PD = glucose-6-phosphate dehydrogenase; 6PGD = phosphogluconate dehydrogenase; PK = pyruvate kinase; GSSG-R = glutathione reductase; HK = hexokinase; LDH = lactate dehydrogenase; GSH = reduced glutathione; APH = acetylphenylhydrazine; ATP = adenosine-5-triphosphate; ADP = adenosine-5-diphosphate; AMP = adenosine-5-monophosphate; 2,3PGA = glycerate-2,3-diphosphate; F-1,6-P = fructose-1,6-diphosphate; PEP = phosphoenolpyruvate; NADH = diphosphopyridine nucleotide, reduced form; RBC = red blood cells; WBC = white blood cells.

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Haematological analysis carried out during the first period in hospital (at 6 years of age) gave the following results: RBC 1,790,000/µl, WBC 5,000/µl haematocrit 15%, remarkable anisopoikilocytosis, autohaemolysis 48 h with glucose 4.95%, without glucose 1.65%, a diagnosis of thalassaemia was made for both father and mother.

Results

In table I the activity of PK, the behaviour of some enzymes most frequently showing genetic alterations and the concentration of some intermediates of glucose metabolism of the propositus are presented. In comparison with normal values, the PK activity is strongly reduced. G6PD and 6PGD activities are normal. The activity of GSSG-R is also greatly reduced and HK activity is moderately increased. GSH displays a high lability after incubation with acetylphenylhydrazine. The concentration of adenine nucleotides does not show significant changes while

Table I. Enzyme activities and some metabolite concentrations in the erythrocytes of the propositus

	Propositus	Normal values
PK	16.2	54.0 ± 5.9
G6PD	52.6	62.0 ± 6.1
6PGD	43.6	49.9 ± 6.0
GSSG-R	5.9	13.2 ± 1.7
HK	5.7	4.4 ± 0.7
GSH before APH	54.5	67.6 ± 6.4
after APH	20.7	58.2 ± 3.0
ATP	0.52	0.70 ± 0.15
ADP	0.25	0.20 ± 0.02
AMP	0.06	0.09 ± 0.02
2,3PGA	11.34	3.9 ± 0.3
F 1,6-P	0.28	0.12 ± 0.03
Triose-Phosphates	0.23	0.10 ± 0.02

Enzymatic activities are expressed in µmol/h/ml RBC at 25 °C. Glutathione concentration in mg/100 ml of packed erythrocytes. Concentration of the other metabolites in µmol/ml RBC. The values for the propositus are the average of three tests carried out on blood samples collected at different periods. The normal values are the mean of ten results obtained from our tests and from other data.

Materials and Methods

Blood was collected with a siliconized syringe and a sufficient amount of heparin was added. After centrifugation the plasma and the white cells were removed by suction the erythrocytes were washed three times with isotonic solution of KCl at pH 7.4 The haemolysates were prepared by treating washed erythrocytes with 2 vol of cold distilled water The ghosts were removed by centrifugation at 10,000 rpm for 30 min All procedures were carried out at +2 °C. For the determination of the metabolites, the washed erythrocytes were resuspended in an equal volume of isotonic solution of KCl and then deproteinized with 3 vol of 10% $HClO_4$ After centrifugation the supernatant was removed from the precipitate and the latter washed twice with 5% HClO 40% KOH was then added to these two reunited supernatants in order to remove $HClO_4$ after centrifugation, the supernatants were neutralized and then brought to volume All procedures were carried out in the cold room at 4 °C. F-1 6-P and the triosephosphates were measured according to SLATER [1], 2,3PGA according to KRIMSKY [2] ATP according to LAMPRECHT [3] ADP and AMP according to ADAM [4] the glutathione content and stability according to BEUTLER *et al.* [5]

PK activity was determined according to the method of BUCHER and PFLEIDERER [6], which was modified by VALENTINE and TANAKA [7] and further adapted by us. In this method, low substrate concentrations are used which permit to obtain more reliable results for PK deficiency [8] the values of absolute PK activity are, however lower than those which are obtained in the presence of saturating concentrations. Measurement mixtures were as follows: Tris 0.045 M, KCl 0.075 M, MgCl 0.01 M, NADH 0.0001 M PEP 0.002 M, ADP 0.0004 M, LDH 1.5 IU/ml erythrocyte haemolysate sufficient to determine optical absorbance variation of 0.01/m.

The activities of G6PD and 6PGD were determined according to KORNBERG and HORECKER [9] and HORECKER and SMYRNIOTIS [10] HK activity according to FROMM and ZEWE [11], glutathione reductase activity according to HORN [12]

GSH content is expressed in mg/100 ml of packed erythrocytes the metabolites contained are measured in μmol/ml of erythrocytes. The activity of the enzymes is expressed in μmol/h/ml of erythrocytes at 25 °C. The affinity constant for phosphoenolpyruvate was obtained first with the usual graphic method of Lineweaver Burk and then reconfirmed using the Olivetti programme 101, which also confirms the linear index of the points obtained by the experiment.

Case Report

At 40 days the patient displayed remarkable paleness and subicterus. He was given a blood transfusion every 20 days up to the age of 2 years. Until the age of 4 years no more transfusions were required but afterwards a blood transfusion was given every 2–3 months. From 1973–1975 biochemical investigations were never carried out close to the time of transfusion.

Haematological analysis carried out during the first period in hospital (at 6 years of age) gave the following results: RBC 1 790,000/µl, WBC 5,000/µl haematocrit 15%, remarkable anisopoikilocytosis; autohaemolysis 48 h with glucose 4.95%, without glucose 1.65%; a diagnosis of thalassaemia was made for both father and mother

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ATP	0.52	0.70 ± 0.15
ADP	0.25	0.20 ± 0.02
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F 1,6-P	0.28	0.12 ± 0.03
Triose-Phosphates	0.23	0.10 ± 0.02

Enzymatic activities are expressed in µmol/h/ml RBC at 37 °C. Glutathione concentration in mg/100 ml of packed erythrocytes. Concentration of the other metabolites in µmol/ml RBC. The values for the propositus are the average of three tests carried out on blood samples collected at different periods. The normal values are the mean of ten results obtained from our tests and from other data.

Table II. Enzyme activities and some metabolite concentrations in the erythrocytes of family members

Family members[1]	PK	G6PD	6PGD	GSH		2,3PGA	F-1 6-P	Triose-phosphates	ATP	ADP	AMP
				before APH	after APH						
Grandmother (father's side), I-1	34.4	–	–	–	–	–	–	–	–	–	–
Grandfather (mother's side), I 2	29 7	44	–	–	–	–	–	–	0.44	0.13	0.09
Grandmother (mother's side), I 3	18.9	49.2	–	–	–	–	–	–	0.36	0 09	0 09
Father II-4	22.6	48.6	6.4	67 1	25.6	5 61	0.15	0.15	0.42	–	–
Mother II-5	21.6	37.5	8.3	81 4	25.9	9 07	0.11	0.16	0.57	–	–
Aunt (mother's side), II-6	48.6	62.9	9 6	86.0	64.0	1 85	0.17	0.06	0.58	0.21	0.06
Uncle (mother's side), II-7	18.9	–	–	–	–	–	–	–	–	–	–
Uncle (mother's side), II-8	18.8	53.4	9.3	65.2	25.7	2.24	0.17	0.08	0.49	–	–
Wife of II-8 II-9	47.8	69 0	13.1	70.6	59 6	1.20	–	0.06	0.49	–	–
Sister III-11	19.9	–	7 7	–	–	5 6	–	–	0.28	0.22	0.14
Female cousin III 12	35.1	–	–	–	–	–	–	–	0.26	0.13	0.06
Male cousin, III 13	34.4	–	–	–	–	4 8	–	–	0.39	0.29	0.15
Female cousin, III 14	26.4	58.6	14 0	60.3	24.6	12.8	0.20	0.07	0.56	–	–
Normal values	54.3	62.5	13.2	67 6	58.2	3.9	0.12	0.10	0.70	0.20	0.09
	±5.9	±6.1	±1 7	±6.4	±3	±0.3	±0.03	±0.02	±0 15	±0.02	±0.02

[1] See genealogical tree (fig. 1). The results for case II-6 are the average results of five tests and those for all the other subjects are the average results of three tests on blood sample collected at different periods. The normal values are the average of ten tests. Enzymatic activities are expressed in µmol/h/ml RBC at 25°C. Glutathione concentration in mg/100 ml of packed erythrocytes. Concentration of the other metabolites in µmol/ml RBC.

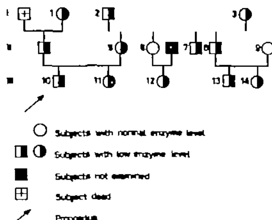


Fig 1 Genealogy of the propositus' family in which members were tested for the PK activity

the concentrations of 2,3PGA, F-1,6-P and of the triosephosphate are increased.

Analysis of the genealogy of the propositus (fig. 1) shows that the defect is present both in the paternal and maternal branches of the family. On the basis of the PK activity the parents, propositus, sister and uncles would seem to be homozygotes, on the basis of the activities and other features shown in tables II and III, the cousins should be considered to be heterozygotes. The family anamnesis showed that clinical manifestations other than in the propositus occurred also in the father and mother who in their youth displayed anaemic syndromes without acute haemolytic episodes. Case II-6, the aunt on the mother's side, with a normal activity is particularly interesting. Table II shows the enzymatic activities of PK, G6PD 6PGD and the concentrations of some metabolites in the relatives of the propositus. A remarkable heterogeneity of results is seen, as many other authors have already observed [13–18]. Particular attention deserves case II-6 in whom all the parameters are normal with exception of a diminution of 2,3PGA.

In the propositus and in some of his relatives we also evaluated the affinity constants for the PEP and the thermoactivation of PK (table III). The changes in affinity constants, such as the index modification of the mutant protein, have been given considerable importance by many

Table III Properties of erythrocyte PK of the propositus and some relatives thermoactivation and affinity constant for PEP

Propositus and some relatives[1]	Thermoactivation before incubation	after incubation	variation %	Affinity constant for PEP
Propositus, III 10	16.2	4 8	−70	0.10
Father II-4	22.6	15 8	−30	0.33
Mother II 5	21 6	10.9	−49	–
Aunt (mother's side), II-6	48 6	63 6	+30	0.29
Uncle (mother's side) II-8	18.8	9.9	−47	–
Wife of II-8, II-9	47.8	34 4	−28	0.50
Sister III 11	–	–	–	0.33
Female cousin, III 12	–	–	–	0.16
Female cousin III 14	26.4	21 9	−17	0.13
Normal values	52.5	70.8	+34	0.35±0.13

[1] See genealogical tree (fig. 1). The thermoactivation was performed by incubating the haemolysate for 90 min at 55 °C. The values are expressed in µmol/h/ml RBC. For the subjects under examination the values are the average of four tests. The normal values are the average of ten results obtained from our tests and from other data.

authors [8, 19–29] but we have found that these changes do not occur in all subjects. A more reliable index of the modification of the protein is obtained from the thermoactivation All subjects examined with the exception of case II-6 (the aunt on the mother's side) showed a decrease in the specific activity while normal subjects are characterized by an increase.

Discussion

The deficiency of PK first demonstrated by VALENTINE *et al.* [30] which is most common as that of G6PD is transmitted as a recessive autosomal condition Research carried out in recent years has, however shown that anaemias due to the defect of PK are characterized by a vast heterogeneity in the clinical and metabolic features, rather than in the genetic aspects [31 32] Anaemia is evident only in the homozygous state [33 34] although clinical manifestations of anaemia in heterozygotes have been reported [14 35 36]

As far as metabolism is concerned, the activity of PK varies in different subjects, as do also the characteristics of this enzyme and the behaviour of other enzymes and metabolites. It has been noted that the increases in the activity of hexokinase or in the triose-phosphate and F-1,6-P content, which were also seen by us, could represent a mechanism which compensates for the metabolic deficiency caused by the genetic defect. This is achieved by means of an increased glucose uptake or by means of a regulatory activity in the phosphorylate compounds. Much importance has also been given to the behaviour of GSH and GSSG-R [37–39] changes at this stage can in fact produce modifications in the erythrocyte reductive state and hence lead to the so-called oxidative haemolysis of Jacop and Jandle.

The cases reported by us are interesting both because they concern two family groups related to each other and because they confirm the heterogeneity of the manifestations. The nature of the defect is variable biochemically clinically and genetically Even in the most severe cases, the level of PK activity generally permits the enzyme to function metabolically and in addition, by means of the Rapoport Luebering cycle, a possible deficiency in the resynthesis of ATP can be compensated for Considering these facts we are inclined to join all those who maintain that anaemias due to PK deficiency are complex forms in which the enzyme defect is only one of the symptoms. Because of the complexity of the problem, therefore, particular attention must be given to possible modifications in the reductive state of the erythrocyte systems.

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Familial Thrombosis Due to Antithrombin III Deficiency in a Greek Family

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Key Words. Antithrombin III deficiency · Coagulation · Familial thrombosis · Heparin cofactor · Thrombosis

Abstract. A Greek family with hereditary antithrombin III (AT III) deficiency associated with venous thrombosis is reported. 5 members of the family were affected. In these patients, AT III and heparin cofactor activities were decreased. Immunoreactive AT III showed a positive correlation to both AT III and heparin cofactor activities. α_2-Macroglobulin and α_1-antitrypsin were normal. The pattern of inheritance of the defect is autosomal dominant.

Antithrombin III (AT III) is the main physiological thrombin inhibitor responsible for 75% of the total plasma AT activity [1]. AT III inhibits also activated factor X [34] and factor VII [26] and inactivates plasmin, trypsin and chymotrypsin [3]; moreover, AT III has a potent heparin cofactor activity [2, 34]. Recently, the report of a number of families with hereditary AT III deficiency with a high incidence of venous thrombosis [12, 19, 25, 30] pointed to the important biological role of AT III in blood fluidity. In the present study, inherited AT III deficiency associated with venous thrombosis is described in a Greek family.

Family Report (table I, fig. 1)

The propositus (fig. 1, III-5) is a 28-year-old male who was in excellent health until September 1973 when he was admitted into the hospital because of 'encephalitis'. During recovery, he developed thrombophlebitis of the right leg and pul-

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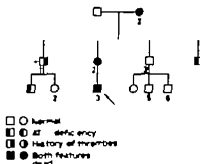


Fig. 1. Pedigree of the family studied.

20 min at 1,200 *g* +2 °C. Serum was obtained from blood clotted in glass tubes and incubated for 2 h at 37 °C prior to centrifugation. The samples were stored at −25 °C. Plastic syringes and siliconized tubes were used.

For evaluation of platelet function, the following tests were performed: platelet count [9], bleeding time [16], platelet adhesiveness [22], platelet aggregation with collagen, ADP and adrenalin [7, 21], ADP release from platelets [33] and platelet factor 3 availability [27]. The following coagulation and fibrinolysis tests were applied: prothrombin, thrombin and partial thromboplastin times [10], fibrinogen [23], factor V [29], factor VIII [15], factor X [11], euglobulin clot lysis time [31], antiplasmin and antiurokinase activities [8], plasminogen [5] and fibrin/fibrinogen degradation products [20].

AT III activity was measured using the techniques of ABILDGAARD *et al.* [4], BIGGS *et al.* [6] and VON KAULLA and VON KAULLA [32]. The method of ABILDGAARD *et al.* [4] uses diluted defibrinated plasma and thrombin. After 3 min of incubation at pH 8.0, the thrombin activity is determined using standard adsorbed bovine plasma. The technique of BIGGS *et al.* [6] utilizes 60 min incubation. The residual thrombin activity is determined using standard fibrinogen solution. In the method of VON KAULLA and VON KAULLA [32], undiluted serum is incubated with thrombin for 5 min. Aliquots from the incubation mixture were added to standard fibrinogen solution and the clotting times were recorded.

AT III concentration in plasma was measured immunochemically by the MANCINI [18] technique using monospecific human AT III antiserum supplied by Behring Diagnostica.

Heparin cofactor activity was determined by the method of EGEBERG [12]. 0.2 ml samples of fibrinogen solution (100 mg/100 ml), heparin (5 units/ml) and plasma (in various saline dilutions) were mixed and incubated for 3 min at 37 °C, after which 0.2 ml of thrombin solution (30 units/ml) was added and the clotting times were recorded; the heparin cofactor activity was determined from the clotting times of control plasma.

The α_2-macroglobulin and α_1-antitrypsin were measured immunochemically using M-Partigen (Behring) plates. For the *in vitro* heparin tolerance test the technique of GODAL [14] was applied.

Table I AT III studies

Number in family pedigree	Age/Sex	Venous thrombosis	AT III levels plasma coagulation tests, % [4]	[6]	serum coagulation test [32], sec	plasma immunoassay [18], %	Heparin cofactor [12], %	α_2-Macroglobulin %	α_1-Antitrypsin %
I 2	70 F	+	46	50	6.1	38	47	105	134
II 2	48 F	+	55	56	7 1	43	61	80	128
II 3	43 M		108	106	35 0	72	96	100	102
II-4	36 M		52	50	7 0	45	56	78	125
III 1	17 M		60	58	–	55	66	94	132
III ..	14 F		112	110	44.0	112	115	98	113
III 3	28 M	+	51	54	5.4	42	48	92	128
III-4	17 F		125	1..8	72.0	130	102	–	–
III 5	12 M		110	100	34 0	106	110	–	–
III-6	6 M		110	112	37.5	116	100	–	–
Normal values									
Mean			102	100	39 8	101	103	96	108
±SD			±15.3	±15 1	±11.9	±13	±16.2	±19 4	±21 1
Range			70–128	72–128	16–75	75–125	72–130	50–150	50–150

monary embolism. 3 months later he developed a new episode of pulmonary embolism together with thrombosis of the left renal vein and of the superficial veins of the right arm. He was treated initially with heparin and subsequently with oral anticoagulants without evidence of new thrombotic phenomena.

Subject I 2 had a history of recurrent thrombophlebitis of both legs, which first appeared after the delivery of her first child at the age of 20. During the last 10 years she had recurrent swelling and pain of both legs.

Subject II 1 died at the age of 36, death has been attributed to thrombosis of mesenteric veins.

Subject II 2, a 48-year-old female, developed thrombophlebitis of both legs after the delivery of her first child at the age of 21 Since then she has been complaining of pain and swelling of both legs.

Subject II-4, a 36-year-old male, presents marked varices in both legs without a history of venous thrombosis.

Material and Methods

Plasma was obtained fasting by collecting 9 vol of venous blood into 1 vol of trisodium citrate 3.8%. Immediately after drawing, the blood was centrifuged for

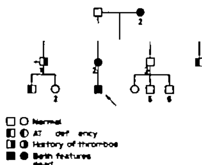


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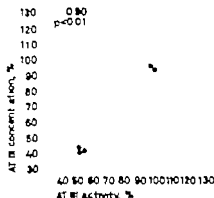


Fig. Relationship between plasma AT III concentration measured by immunoassay and the AT III activity assayed by coagulation tests.

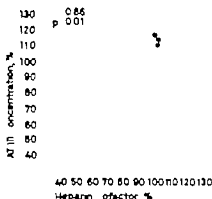


Fig. 3. Relationship between plasma AT III concentration measured by immunoassay and heparin cofactor activity

Results

The propositus was extensively studied for evidence of haemostatic abnormalities. All tests performed for the evaluation of platelet function, coagulation and fibrinolysis ('Material and Methods') gave results within normal limits.

Antithrombin studies. Table I shows the results of the AT III measurement in the 10 members of the family studied. AT III activity in plasma and serum as well as AT III concentration in plasma were found to be markedly decreased in 5 members of the family. The results ob-

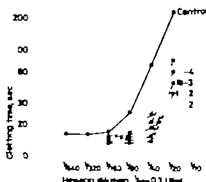


Fig. 4. Heparin tolerance test (in plasma-thrombin system).

tained by the technique of ABILDGAARD *et al.* [4] (short incubation) and BIGGS *et al.* [6] (long incubation) were almost identical. The levels of AT III concentration, obtained immunochemically, were slightly lower compared to the coagulation assay. In the technique used for estimation of serum AT III the results are expressed in clotting times and are not comparable to those obtained by the other methods. The separation of the deficient members of the family by this technique was even easier.

The heparin cofactor activity was found to be decreased in 5 patients, the same with AT III deficiency. The values of plasma immunoreactive AT III showed a significant positive correlation to both plasma AT III activity and heparin cofactor activity (fig. 2, 3). The same correlation existed in the other 5, apparently normal, members of the family as well as in 10 controls. The α_2-macroglobulin and α_1-antitrypsin were within normal limits in all patients studied.

The heparin tolerance of the patients with AT III deficiency was increased (fig. 4).

Discussion

Hereditary deficiency of AT III has been reported in several families [12, 19, 25, 30]. In all of them, the majority of the affected individuals were unusually susceptible to thromboembolic disease. In the present study of a Greek family, 5 out of the 10 members studied had AT III deficiency; 3 of them have a history of severe venous thrombosis and

another has signs of venous insufficiency in both legs The fifth deficient patient was a young man free as yet of any thrombotic complication

The pattern of inheritance of the disorder in our family as well as in all so far reported families, was autosomal dominant The incidence of the dominant character of inheritance suggest that it is extremely low On the other hand, many cases may escape attention because AT III assay is rarely performed routinely in most laboratories

All methods used demonstrated the AT III deficiency in all 5 patients but the difference from the normal was more striking by estimating serum AT III A recently reported [24] family with deficiency of plasma progressive thrombin inactivation but normal immunoreactive AT III points however to the need of the performance of both coagulation and immunochemical tests.

The antithrombin effect of heparin is mediated through a plasma factor the heparin cofactor or antithrombin II There is evidence that AT III and heparin cofactor activities arise from the same molecule [2 34] Heparin cofactor was found decreased in families with AT III deficiency [12 19 24] In the present study a highly significant positive correlation of AT III concentrations (immunoassay) with AT III activity (coagulation assay) and heparin cofactor was found These results, are in accordance with similar earlier observations [12] and support the molecular identity of AT III and heparin cofactor

Apart from AT III two more proteins, α_2 macroglobulin and α_1 antitrypsin are known to act as antithrombins [13 17 28] The biological significance of them in the prevention of thrombosis, is obscure. Both were found normal in the serum of patients with AT III deficiency This finding, appears to support the assumption that their biological role if any is minor

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Tetraploid Cell Line in a Girl with Acute Leukaemia

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The Hospital for Sick Children, London

Key Words Cell culture Cytokinetics DNA synthesis Karyotype Leukae-
Tetraploid blast cells

girl with atypical acute leukaemia the malignant cell line in peri-
marrow during three relapses was found to be tetraploid.
proliferative than those usually present in acute leu-
of the blast cells distinguished them from oth-
from this patient and from the blast cells found
tic leukaemia.

in acute leukaemia of childhood are
aberration, when it occurs, does not
of changes have been observed
[13 19]
a 7½-year history of an acute
to be tetraploid.

tis, extensive bruising
19×10^9/litre, platelets
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Tetraploid Cell Line in a Girl with Acute Leukaemia

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Key Words. Cell culture · Cytokinetics · DNA synthesis · Karyotype · Leukaemia · Tetraploid blast cells

Abstract. In a girl with atypical acute leukaemia the malignant cell line in peripheral blood and bone marrow during three relapses was found to be tetraploid. The abnormal cells were more proliferative than those usually present in acute leukaemia. The tetraploid DNA content of the blast cells distinguished them from other cells in blood and bone marrow from this patient and from the blast cells found in other instances of acute lymphoblastic leukaemia.

Abnormal chromosome patterns in acute leukaemia of childhood are well recognised [17]. Chromosomal aberration, when it occurs, does not appear to be specific and a great variety of changes have been observed [16]. Polyploidy has been described in malignant lymphoma [13, 19].

The patient presented here is a girl with a $7^1/_2$-year history of an acute leukaemia whose malignant cells were observed to be tetraploid.

Case Report

A $9^1/_2$-year-old Irish girl was seen initially with polymyositis, extensive bruising over the legs and splenomegaly. Haemoglobin 7 g/dl, WBC 19×10^9/litre, platelets 30×10^9/litre. Differential white cell count: neutrophils 48%, monocytes 2%, lymphocytes 48%, and blast cells 2%.

A diagnosis of acute stem cell leukaemia was made from the findings in the bone marrow in which nearly all cells were large and immature with scanty cytoplasm and clefted nuclei. Remission was induced with prednisone and 6-mercaptopurine (6MP) and maintained with 6MP for 6 months. Eleven months after the initial

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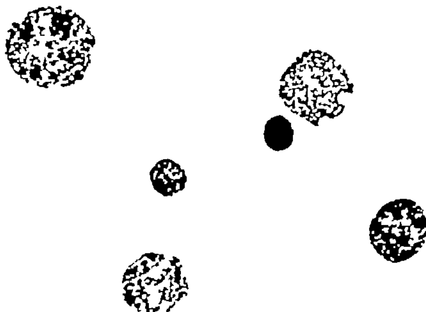


Fig 1 Peripheral blood film showing the characteristic large blast cell

Table I. ^{3}H TdR uptake of circulating blast cells during the treatment of first relapse

Days	WBC/µl	Blast cells %	LI %	Total blast cells/µl	Total labelled blast cells/µl
0 (initial)	12,400	17	17	2,100	358
2	15,800	17	16	2,700	432
5	20,400	25	8	5,100	408
7	30,800	52	10.5	16,100	1,680
8	23,000	55	10.5	12,650	1,328
11	21,200	37	8.5	7,840	666
13	10,600	25	7.6	2,650	208
17	3 100	11	8	341	10
20	3,060	2	1.0	61	0.6
22	2,500	4	1.0	100	1
4	1,320	0	0	0	0

admission she was found to have splenomegaly 6MP was restarted and continued for 6 months

The patient remained well with no chemotherapy for 6 years she then presented with bleeding purpura and ecchymoses. There was hepatosplenomegaly and a large mass rising up to the umbilicus, which was thought to be ovarian in origin. At this time the haemoglobin was 11 1 g/dl and WBC 12×10⁹/litre of which 17% were large blast cells with scanty cytoplasm and 1–? nucleoli. These cells were present in the marrow and similar to those seen 7 $^1/_2$ years previously Treatment with prednisone and 6MP was again instituted. After 1 month the clinical and haematological findings became normal. She relapsed 2 months later when the circulating blast cells and bone marrow appearances were identical to those seen previously The third relapse 6 months later was complicated by septicaemia and she died shortly after

Methods

Cytokinetic study Bone marrow or peripheral blood samples were incubated separately with 1 µCi/ml of ^{3}H TdR and 5 µCi/ml of ^{3}H/UR. Autoradiographs were prepared and stained using Giemsa 1 000–2,000 blast cells were counted and the number of labelled cells expressed as a percentage.

Short-term culture Heparinised 'buffy coat' preparations from the peripheral blood were incubated with TC199 and AB serum at 37 °C ^{3}H TdR (1 µCi/ml) was added to individual cultures 30 min before termination at 2, 3, 4 6 and 8 h and processed as above.

The DNA content of blast cells was measured by light absorption of Feulgen-stained nuclei using a Deely pattern microdensitometer

Using a combination of microdensitometry and autoradiography the percentages of cells in S, G G and M were determined [1] The cell cycle time was measured using the method of STEEL and BENSTED [21]

Chromosomes were studied in peripheral blood culture and directly on bone marrow

Results

Labelling index peripheral blood In the first relapse, after 6 months remission the blood contained very many large primitive cells with a mean diameter of 15–20 µm and scanty cytoplasm (fig. 1) The uptake of ^{3}H TdR by these circulating blast cells was high (17%) before chemotherapy and was 18.5 % in the final relapse. During the treatment of the first relapse the labelling index of peripheral blast cells was measured every 2–4 days as shown in table I

In figure 2 the labelling index (LI) of blast cells in the peripheral blood in short term culture is shown It is evident that the number of cells

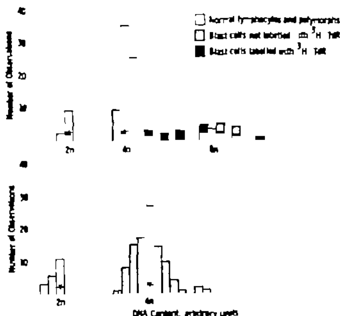


Fig 3 Distribution of nuclear DNA content of blast cells in blood (top histogram) and bone marrow (lower histogram). — Modal average.

Labelling index bone marrow Insufficient marrow was obtained at the time of first relapse for study During the second relapse, when blast cells formed 9% of all nucleated elements, the LI of blast cells was 18%. This decreased to 13% in the subsequent remission and rose again to 47% during the final relapse. Such a high proliferative activity of blast cells has been observed in only a few of the leukaemic children we have studied. The amount of ^{3}H TdR incorporated into blast cells of this patient was much greater than in a normal diploid population. This is shown by comparing the mean grain count over blast cells and myelocytes present on the same slide (table II). It may be that the higher grain count is due to the fact that the cells are larger and have at least twice the DNA values of a normal cell population but the pattern of DNA synthetic activity was unlike a normal population in that there was no correlation between the grain count and the DNA content of the blast cells. This means that there is no definite change in the rate of DNA synthesis as the cell proceeds

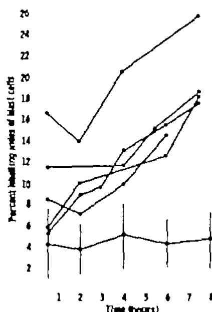


Fig 2 Comparison of the proliferative activity (LI) of tetraploid cells and other leukaemic blast cells in short term culture. Five observations taken on separate occasions showing the increase in the LI of tetraploid cells compared with the mean and standard deviation of blast cells from 10 children with acute lymphoblastic leukaemia (lower curve)

Table II Comparison of the labelled myelocytes and blast cells in a single autoradiograph

	Individual grain counts												Cells counted
	120	110	100	90	80	70	60	50	40	30	20	10	
Blast cells													
No	1	–	8	7	6	7	11	11	4	3	2	0	60
%	2		13	12	10	12	18	18	7	5	3	0	
Myelocytes													
No.								7	10	8	7		32
%								22	31	25	22		

in DNA synthesis increased markedly during the 8 h of culture. The rise in LI of blast cells in culture is unlike that studied in acute lymphoblastic leukaemia in which the number of proliferative cells remains unchanged as illustrated in figure 2

Table III Chromosome studies during remission and relapse

State of disease	Sample	Number of chromosomes												Polyploid
		<46	46	80	81	86	87	88	89	90	91	92	100	%
Relapse	blood		10		1	1		3	1	4	1	18	1	64 0
During induction of remission (2 weeks therapy)	blood	3	28											
Terminal relapse	bone marrow		1		1	1		1	1	1	1	21		36 0

traploid. In the final relapse cells were mainly tetraploid with a spread of cells around 4n indicating some heterogeneity

Chromosome analysis Chromosome studies during the first relapse showed 64 of peripheral blast cells were polyploid, of which over 60°/ were tetraploid (fig. 4) After treatment with prednisone and 6MP the polyploid and particularly the tetraploid cells disappeared from the blood and the majority of cells had 46 chromosomes. In terminal relapse the te traploid cells were clearly present in the bone marrow (50° table III).

Compartmental analysis The distribution of blast cells in different compartments of the cell cycle was determined by a combination of autoradiography and microdensitometry Examining 1 000 cells, post-mitotic (G) comprised 78.5 while those in DNA synthesis were 17°/. Pre mitotic (G_2) were 4 1 /$_0$ and mitotic, 0 4 /. The S/G ratio is 4 1/1 This is much higher than that usually found in acute lymphoblastic leukaemia and more like that associated with solid tumors [12] The potential doubling time of blast cells using Steel s formula [21] was in the order of 44 h which is shorter than that usually recorded in acute leukaemia [4] The RNA synthetic acti ity of the blast cells was high during the first relapse (about 70°/) and remained at the same level in the final relapse.

Discussion

This patient presented with several atypical features but was considered to be suffering from acute lymphoblastic leukaemia. The course of the disease was unusual in that an easily induced remission was main-



Fig 4 Metaphase plate from circulating blast cell

through the S stage Thus the grain count cannot be used as an indication of a tetraploid state.

DNA content The amount of DNA in peripheral blast cells at the time of the first relapse is illustrated in figure 3 The nuclei of myelocytes and lymphocytes were also measured to obtain the DNA value of normal diploid cells. It is evident that the majority of blast cells have a DNA val ue twice that of normal diploid cells Cells with more than tetraploid DNA content were either in S phase (labelled with ^3H TdR) or in G_2 Analysis of nuclear DNA content of blast cells from the bone marrow in the second relapse showed two populations of which the majority were te

longed administration of busulphan to young rats caused inhibition in mitosis giving rise to tetra- and polyploidy in lens epithelial cells [9]. The morphology of bone marrow cells of our patient at diagnosis was similar to that studied in relapse and thus the large cells were probably tetraploid from the commencement of the disease and it is unlikely that her short course of chemotherapy influenced the modal chromosome number. It is also unlikely that during the prolonged drug-free remission a mutant gene developed. If a suppression in mitosis had given rise to a tetraploid state, this would have affected only one generation as there was no accumulation of blast cells in 8 and 16n. An external influence seems unlikely to have modified the malignant process in this patient and we consider she had a persistent tetraploid cell line from the commencement of her disease, associated with marked sensitivity to cytotoxic therapy and a relatively good prognosis.

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ained without chemotherapy for 6 years after only 1 year of treatment with 6MP alone The proliferative activity of the patient s blast cells was nr nced in culture where the LI rose markedly over a period of 8 h It is ssible that in this culture environment, there was recruitment of cells S phase and the high proliferative activity may not reflect the situa- n vivo Similar *in vitro* enhancement has been observed in cells from nts with glandular fever [6] The proportion of blood blast cells ac synthesising DNA is much greater than that usually observed in ute lymphoblastic leukaemia [7 10 18] Labelling indices of over 30% are uncommon in relapse of acute leukaemia [8 14] The pattern of proliferative activity in the blast cells seen in this patient is similar to that of a child with widespread lymphosarcoma [unpublished observation] Burk itt s lymphoma [5] and reticulum cell sarcoma [12] The leucoerythroblastic blood picture found in this patient together with the densely cellular marrow and soft tissue involvement suggests the possibility of lymphosarcoma rather than acute lymphoblastic leukaemia.

The ratio of the number of cells in the S and G_1 has been used as an other parameter of proliferative activity The S/G_1 ratio of 4 1/1 in this patient is similar to that found in reticulum cell sarcoma [12] and Burk itt s lymphoma [5] There is no accumulation of cells in G_2 and therefore cells with 4n DNA content are a tetraploid line

The DNA content of leukaemic cells in this patient remained tetra ploid in each relapse studied although tetraploid cell lines are not a feature of acute leukaemia Hypotetraploidy and endoreduplication has been reported in a few patients with acute leukaemia [2, 11] but in none were the tetraploid cells prominent. Tumours with tetraploid features have been noted with greater frequency in reticulum cell sarcoma [12] and there appears to be some correlation between the modal chromosome number and clinical presentation of the disease. Patients with tetraploid reticulum cell sarcoma had more localised disease were mostly female and responded favourably to treatment Polyploid tumours in the bladder show higher chromosome numbers in the most malignant forms [20] Tetraploidy is a feature of normal liver and bladder cells in man.

Polyploidy may develop in several ways of which endoreduplication has been observed in acute leukaemia [2] and cultures of mammalian cells [15] Another mechanism is mitosis with a single spindle giving rise to cells which can return to interphase with a tetraploid level of DNA The development of tetraploid, octaploid and hexadecaploid nuclei has been observed in fibroblast cultures in the presence of busulphan [3] Pro-

Acta haemat. 57 65–73 (1977)

Effect of Blood Transfusion on the Immune Response of Children with Thalassaemia

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Key Words. Blood transfusion · Immune response after blood transfusion · Immune response in thalassaemia · Thalassaemia

Abstract. The effect of blood transfusions on the immune response of 46 thalassaemic children was studied. Cell-mediated immune response was evaluated by performing skin tests to specific (streptokinase-streptodornase and candidin) and non-specific (dinitrochlorobenzene and phytohaemagglutinin) antigens. Antibody response to a specific antigen (tetanus toxoid) was estimated by measuring the tetanus antitoxin titre before and after vaccination. No gross impairment of cell-mediated immunity was elicited. The larger proportion of negative phytohaemagglutinin skin tests found in thalassaemic patients does not necessarily suggest a cell-mediated immunity impairment, since this skin reaction is also affected by other factors, especially the inflammatory skin response. The transfused antibodies may inhibit the recipient's sensitization and primary immune response to the homologous antigen, especially when the antibody level in the transfused blood is high, whereas the secondary immune response is not affected.

Antigenic factors as well as antibodies may be transferred to a patient receiving blood transfusion. SCHECHTER *et al.* [9] believe that administration of even a small amount of blood causes a definite immunological stimulation in the recipient, expressed by the appearance of atypical activated lymphocytes in the circulation 1 week after the transfusion. It would seem, therefore, that it might be interesting to study the immune response of subjects receiving regularly blood transfusions for therapeutical purposes. Such subjects are those suffering from thalassaemia and a study regarding thalassaemic patients would seem more justified as these patients are more often affected from various infections than other people.

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0.1 ml of varidase solution were injected intradermally and the result was read after 48 h. *(c) Candidin skin test.* 0.1 ml of *Candida albicans* intradermal testing solution was injected intracutaneously and the result was read after 48–72 h. Skin tests were characterized as positive when an induration of >5-mm diameter was present at the site of injection.

Tetanus antitoxin titration before and after vaccination. The antitoxin level in the serum before and after vaccination against tetanus was measured by haemagglutination using a slightly modified technique described by FULTHORPE [4]. Tetanus toxoid (XTO9 Wellcome) and tetanus standard antitoxin (XAO6, Wellcome) were used. Fresh heparinized sheep blood was used as source of erythrocytes obtained on the day of the experiment. The antitoxin titration was performed in 46 patients and in 23 controls. It was also performed in 15 randomly selected blood vials used for transfusion of thalassaemic children who had never been previously vaccinated against tetanus.

Biochemical Estimations

Serum antistreptolysin O (ASTO) titre was measured in all thalassaemic children and controls, who had negative SK-SD skin test, according to standard methods.

Results

Cutaneous reactivity to various antigens. The results of cutaneous reactivity to DNCB, PHA, SK SD and candidin are listed in table I. No difference in the cutaneous reactivity to DNCB and candidin was observed between the patient (93.2 and 45.7%) and the control groups (92.7 and 51.6% positive skin tests, respectively). The PHA and SD-SK skin tests

Table I. Results of the skin tests to DNCB, PHA, SK SD and candidin of children with thalassaemia and controls

Number of patients	Children with thalassaemia major								
	DNCB		PHA			SK-SD		candidin	
	+	–	+	±	–	+	–	+	–
44	41	3	13	5	23	19	22	16	19
Number of controls	Controls								
48	38	3	30	8	3	36	12	16	15
χ^2	0		22.4			6.89		0.25	
p	1		< 0.001			<0.01		>0.60	

Induration diameter = 5 mm.

VALASSI ADAM [12] measured the immunoglobulin levels in children with thalassaemia. She did not find any significant difference in any of the immunoglobulins between patients and age matched controls, unlike SEITANDIS *et al.* [10] who found high immunoglobulin levels in splenectomized thalassaemic patients. SEITANIDIS *et al.* [11] also measured the third component of the serum complement and did not find any significant difference between patients and age matched controls. No information is available regarding the cell mediated immunity in thalassaemic children. On the other hand, no data are available regarding the effect of the transfused antibodies on the recipient's primary immune response to a homologous externally induced, antigen.

These reasons led us to study the cell mediated and humoural immune response in a group of children suffering from β-thalassaemia major.

Materials and Methods

Subjects Studied

46 children (27 males and 19 females) with thalassaemia, aged between 6 months and 13 years, were studied. 35 of them had been transfused for at least 20 times when the study began. The interval between two transfusions was, in most of the patients, 1 month. None of the patients had been splenectomized. All the skin tests and vaccines were performed with the permission and co-operation of the parents of the children.

Collection of Blood Samples

The first specimen of blood was obtained from all patients at the time they were admitted for transfusion. At the same time, a careful history of the previously done vaccines was taken from the parents and then a vaccination against tetanus (adsorbed tetanus toxoid Glaxo) was performed. The second blood specimen was obtained 1 month later that is when the patients came in for their next transfusion. The same procedure, as far as sampling and vaccination is concerned, was followed with the matched for age and sex controls. Sera were coded and stored at –20 °C.

Immunological Studies

Cutaneous delayed hypersensitivity to specific and non-specific antigens. Skin tests to various antigens were performed in 44 patients at the time of collection of blood samples. These included skin test to dinitrochlorobenzene (DNCB) according to the technique described elsewhere [7] and intracutaneous tests to phytohaemagglutinin (PHA, Wellcome) streptokinase-streptodornase (SK-SD 'Varidase' Lederle) and candidin (Bencard) performed as follows: *(a) PHA skin test*: 0.02 mg of PHA in 0.1 ml of normal saline were injected intradermally and the result was read after 24 h, *(b) SK SD skin test*: 10 U of streptokinase and 25 U of streptodornase in

0,1 ml of varidase solution were injected intradermally and the result was read after 48 h; *(c) Candidin skin test.* 0.1 ml of *Candida albicans* intradermal testing solution was injected intracutaneously and the result was read after 48–72 h. Skin tests were characterized as positive when an induration of a>5-mm diameter was present at the site of injection.

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	+	−	+	±	−	+	−	+	−
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Induration diameter = 5 mm.

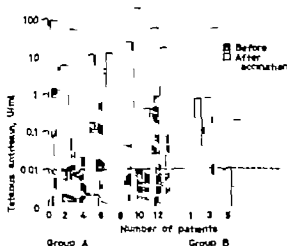


Fig 1 Antibody response to tetanus toxoid in children with thalassaemia completely (group A) or incompletely (group B) vaccinated against tetanus.

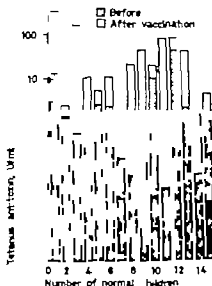


Fig 2 Antibody response to tetanus toxoid in normal children previously vaccinated against tetanus.

were found to be negative in a significantly larger proportion of patients (54 7 and 53 6%) in comparison to the controls (7.3 and 25% respectively)

Antibody response to tetanus toxoid antigen The patients were divided into three groups (A, B and C) according to their vaccination history

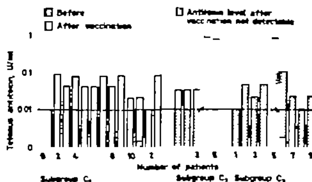


Fig 3. Antibody response to tetanus toxoid in children with thalassaemia never vaccinated against tetanus (group C).

against tetanus in their infancy and to the antitoxin titre found in the first serum sample at the time vaccination was performed. *Group A* included 13 children who had been completely vaccinated against tetanus during infancy. A high antitoxin titre was found and this increased 1 month after the vaccination of the present study (fig. 1). In the matched for age and sex control group the antitoxin level was also found to be high before and after the vaccination (fig. 2). *Group B* included 6 children who had been incompletely vaccinated against tetanus during infancy. Non-detectable antitoxin levels were found before vaccination, whereas were found to be high 1 month thereafter (fig. 1). Matched for age and sex controls were not available for this group of patients. *Group C* included 27 children who had never been immunized against tetanus. The children of this group were divided into three subgroups (C_1, C_2 and C_3) according to the serum antitoxin levels found before and after vaccination of the present study (fig. 3). *Subgroup C_1* included 13 children who had no detectable antitoxin level in their serum before vaccination. In all of them the antitoxin titre was found to be equal with or above the critical protective level (0.01 antitoxin U/ml serum) 1 month after vaccination. Matched for age and sex controls had a very similar antibody response (fig. 4). *Subgroup C_2* included 6 children who had an antitoxin titre equal with or above the critical protective level. In all children of this group the antitoxin titre was found to be higher 1 month after the vaccination. *Subgroup C_3* included 9 children who had a similar to subgroup C_2 antitoxin level before



Fig 4 Antibody response to tetanus toxoid in normal children never vaccinated against tetanus.

vaccination but this was found to be unchanged, lower or not detectable 1 month thereafter

Tetanus antitoxin titre in the transfused blood. In 5 out of the 15 samples taken from transfused blood units, the antitoxin titre was found to be above the critical protective level. All these blood units had been transfused to children included in the C_3 subgroup

Antistreptolysin O Titre In 6 out of 17 patients with negative SK-SD skin test, the ASTO titre was found to be elevated (>150 U/ml), whereas it was found to be normal (<125 U/ml) in all controls with negative SK SD skin test.

Discussion

Cell Mediated Immunity

The study of cutaneous reactivity to various antigens did not reveal any gross impairment of cell-mediated immunity in thalassaemic children of the present series. The cell mediated immune response can be evaluated using skin tests to specific and non-specific antigens. Contact sensitization to DNCB is a more reliable and exact test for cellular immunity for the following reasons. (a) previous exposure to this substance is not needed (b) over 95% of normal subjects can be sensitized to DNCB [3] and (c) circulating antibodies do not develop with sensitization [13] Skin re-

action to PHA is morphologically and histologically similar to the delayed type hypersensitivity reactions [1]. However, PHA skin test is not considered to be absolutely specific for evaluating cellular immune competence, since a negative skin test may result from abnormalities in the inflammatory response as well as from specific impairment of delayed type hypersensitivity [2].

In the present series, a significantly larger proportion of negative PHA skin tests was found in thalassaemic children (54.7%) when compared to the control group (7.3%), whereas the proportion of negative DNCB skin tests was approximately the same in both groups (6.8 and 7.3%, respectively). This finding can be due to the fact that PHA is not such a potent irritant and immunogen as the DNCB and the skin reaction to PHA partly depends on normal skin inflammatory response [2, 6]. In thalassaemic children, haemosiderosis of skin and lymphoid organs usually develops and can affect the normal production and function of lymphocytes and macrophages.

The larger proportion of negative SK SD skin test found in thalassaemic children (53.6%) when compared to the controls (25%) might be explained by the passive transfer to these children of antistreptococcal antibodies in an amount that could inhibit their sensitization to streptococcal antigens. In favour of this concept is the following: (1) most of the thalassaemic children in the present series had never suffered from tonsillitis attacks and (2) a high serum ASTO titre was found in 8 out of the 17 thalassaemic children, who had negative SK SD skin test but in none of the controls.

Humoural Immunity

The antibody response to tetanus toxoid in thalassaemic children of the present series was found to be affected by the tetanus antitoxin level found in their serum, due to passive transfer with the transfused blood. It is known that the critical serum protective level of tetanus antitoxin is 0.01 U/ml. Considerable studies have demonstrated that protective antitoxin levels persist in serum of completely vaccinated subjects for over 10 years [8]. Furthermore, a protective level of tetanus antitoxin can be achieved with a single booster dose of tetanus toxoid, even though 10 or more years have lapsed since the primary series [5]. According to these data, the immune response of the children in the present study was characterized to be normal if serum tetanus antitoxin titre was raised to protective levels after vaccination.

Our findings in the three groups of thalassaemic children suggest the following: (1) When a primary series of tetanus toxoid injections had been done in the 1st year of life (group A and B) the antibody response to booster tetanus toxoid dose was normal and approximately the same with the matched for age and sex control group (fig. 1, 2). (2) When immunization against tetanus had never been performed (group C) the antibody response to the vaccination of the present study mostly depended upon the serum level of tetanus antitoxin which had been passively transferred to the patients with the transfused blood. Thus, the antibody response of children included in subgroup C_1 (no detectable serum antitoxin level before vaccination) was normal (fig. 3) and approximately the same with the control group (fig. 4) whereas the antibody response of children included in subgroups C_2 and C_3 (detectable antitoxin level before vaccination) varied widely. In only 5 out of 14 children in these subgroups, the antitoxin titre was further raised after vaccination (subgroup C_2). The remaining 9 children did not develop any antibody response (subgroup C_3). In other words, the presence of the passively transferred antibodies (tetanus antitoxin) to the homologous antigen (tetanus toxoid) inhibited the sensitization and primary immune response in these children. The antibody response was not related to the patients age and to the number of blood transfusion that they had received.

It is concluded that the passive transfer of high levels of antibodies to the transfused thalassaemic patient may result in inhibition of his primary sensitization and development of primary immune response to the homologous antigen whereas it has no effect on the secondary immune response.

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A Simplified One-Step Procedure for the Simultaneous Determination of Complement Receptor Lymphocytes and Lymphocytes with Membrane-Bound Immunoglobulins

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Key Words. Complement Complement receptor lymphocytes Immunoglobulins Lymphocyte immunoglobulins

Abstract A simple one-step procedure for the demonstration of complement receptor lymphocytes (CRL) with complement-coated bacteria (BC) as indicator particles is described. With this assay the percentage of CRL in normal peripheral blood ranged between 6 and 21% (mean 11%) In a separately performed combined assay for lymphocytes with membrane-bound immunoglobulins (M Ig) and lymphocytes, which form rosettes with complement-coated bacteria (BC RFC), four different fractions of lymphocytes could be detected. (M-Ig^+–BC^+ M-Ig^+–BC^- M Ig^-–BC^+ M Ig^-–BC^-). These results suggest that the subpopulation of lymphocytes with complement receptor sites overlaps with, but is not totally identical with the lymphocyte subpopulation bearing Ig on the surface.

In recent years a great deal of evidence has accumulated indicating, that among others, receptors for modified C3 exist on lymphocytes, macrophages, granulocytes and platelets in several mammalian species [1 7 9 11 14 16] Complement receptor lymphocytes (CRL) are usually demonstrated in a rosetting procedure by incubating lymphocytes with red cells (E) coated with antibody (A) and complement (C) Besides erythrocytes also bacteria coated with antibody and complement as indicator cells [5] and complement-coated zymosan beads [8, 13] were used

In this study an attempt is made to develop a simple one-step method for the determination of CRL, which can be combined with the direct immunofluorescence technique and therefore can be easily used for the

simultaneous demonstration of complement receptor sites and immunoglobulins on the surface of lymphocytes.

Material and Methods

Lymphocytes. Blood from 23 healthy adults and 10 children with chronic tonsillitis was collected in heparin and lymphocytes were isolated according to BØYUM [3]. The contamination with α-naphthyl-acetate esterase-positive monocytes was about 10%.

Preparation of complement-coated bacteria (BC). 0.1 ml of heath-killed bacteria (B) *E. coli* 0125B:15 or *Salmonella* (4–8×10⁹ /ml), suspended in veronal buffered saline pH 7.4 with 0.1% gelatine (VBS-GEL) were incubated with 0.1 ml of fresh or stored at –70 °C human sera (C_{hu}) or with guinea pig sera (C_{gp}) in several dilutions at 37 °C for 60 min. Controls were performed by incubating the bacteria with medium or heat-inactivated serum. In 7 experiments 0.01 M ethyleneglycoltetraacetate or 0.01 M ethylenediaminetetraacetate was added to human fresh sera (C_{hu} EGTA, C_{hu} EDTA). In one instance C_{hu} EDTA serum was absorbed with *E. coli* in the cold overnight and resupplemented next day with $MgCl_2$ (C_{hu} ABS). *E. coli* bacteria were incubated with all these sera. Afterwards the bacteria were washed three times and resuspended in VBS-GEL.

Demonstration of C3 bound to the bacterial surface. BC were stained with fluorescein isothiocyanate (FITC) labelled goat anti-human beta 1 C/beta 1A (Behringwerke, batch No. 619D) or FITC-labelled rabbit anti-guinea pig beta 1 C/beta 1A.

Rosette formation of lymphocytes with complement-coated bacteria (BC_{gp}-RFC). 4–8×10⁸ bacteria previously incubated with 1:2 dilution of C_{gp} were mixed with 2–4×10⁶ lymphocytes and incubated for 30 min at 37 °C under continuous rotation. After incubation the lymphocytes were checked for rosette formation. Rosetted (three and more bacteria attached to the lymphocyte membrane) and unrosetted lymphocytes were counted in several fields. A total of 200 lymphocytes was counted for each test.

Demonstration of EAC rosette-forming cells (EAC-RFC). This test was performed essentially according to MICHLMAYR and HUBER [14]. Briefly, EAC-RFC were determined using sheep erythrocytes, anti-Forssman antibody and mouse complement.

Demonstration of lymphocytes with membrane-bound immunoglobulins (M-Ig-Ly). The immunofluorescence staining technique for the demonstration of M-Ig-Ly was performed essentially as previously described [10]. The rabbit anti-human $F(ab)_2$ conjugate labelled with FITC was prepared according to HEUSSER [6] and extensively absorbed with normal guinea pig serum covalently bound to agarose beads according to AXEN *et al.* [2].

Combined assay for M-Ig-Ly and BC_{gp}-RFC. Lymphocytes were stained with the anti $F(ab)_2$ conjugate and then rosetted with BC_{gp}. Afterwards, lymphocytes were simultaneously examined for both, rosette formation in phase contrast and for fluorescent surface staining in epi-illumination [15].

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Rosette formation of lymphocytes with complement-coated bacteria (BC_{gp}-RFC). $4–8 \times 10^{9}$ bacteria previously incubated with a 1:2 dilution of C_{gp} were mixed with $2–4 \times 10^{6}$ lymphocytes and incubated for 30 min at 37 °C under continuous rotation. After incubation the lymphocytes were checked for rosette formation. Rosetted (three and more bacteria attached to the lymphocyte membrane) and unrosetted lymphocytes were counted in several fields. A total of 200 lymphocytes was counted for each test.

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A Simplified One-Step Procedure for the Simultaneous Determination of Complement Receptor Lymphocytes and Lymphocytes with Membrane-Bound Immunoglobulins

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In this study an attempt is made to develop a simple one-step method for the determination of CRL, which can be combined with the direct immunofluorescence technique and therefore can be easily used for the

Table II Combined assay for M-Ig-Ly and BC_{gp}-RFC (33 experiments)

	Mean, %	Range, %
$M\text{-}Ig^{+}\text{–}BC_{gp}^{+}$	8.33	2 20
$M\text{-}Ig^{+}\text{–}BC_{gp}^{-}$	13.48	4–30
$M\text{-}Ig^{-}\text{–}BC_{gp}^{+}$	6.61	20
$M\text{-}Ig^{-}\text{–}BC_{gp}^{-}$	71.09	55–82
$M\text{-}Ig^{+}$–Ly total	21.81	2 30
BC^{+}–Ly total	14.94	2–20
$M\text{-}Ig^{+}$ and BC total	28.42	

periments) 4 different types of lymphocytes could be distinguished. 2–20% (mean 8.33 %) of peripheral blood lymphocytes were detected with both markers on their membrane ($M\ Ig^{+}\text{–}BC_{gp}^{+}$). The percentage of lymphocytes carrying only membrane-bound immunoglobulins ($M\ Ig^{+}\text{–}BC_{gp}^{-}$) ranged from 4 to 30% (mean 13.48%). Lymphocytes with complement receptors only ($M\text{-}Ig^{-}\text{–}BC_{gp}^{+}$) ranged from 2 to 20% (mean 6.61 %). Lymphocytes without complement receptors or membrane-bound immunoglobulins ($M\text{-}Ig^{-}\text{–}BC_{gp}^{-}$) were found to be between 55 and 82% (mean 71.09%). The total number of lymphocytes bearing complement receptors was found to be in the range of 2–20% (mean 14.94 %), and the total number of those bearing Ig ranged from 2 to 30% (mean 21.81 %). A total of 28.42% (mean percentage) of lymphocytes showed either complement receptors or membrane-bound immunoglobulins or both. Calculating the percentages of the single fractions belonging to this group, it is evident, that 47% belong to the fraction $M\ Ig^{+}\text{–}BC^{-}$ whereas 31 % carry both markers ($M\ Ig^{+}\text{–}BC_{gp}^{+}$) and 23% were found to carry only complement receptors ($M\text{-}Ig^{-}\text{–}BC_{gp}^{+}$).

Discussion

The above-described method for the determination of CRL with complement coated bacteria is simple and rapid. C3 can be bound to bacteria (*E. coli* 0125B 15 and *Salmonella*) by incubating them with either fresh normal human sera or with fresh guinea pig sera. Complement-coated bacteria can then be used for the demonstration of complement receptor lymphocytes. Bacteria coated with C guinea pig were used in our 33 experiments, although also C_{hu} can be used. Since after the

Table I BC_{gp}-RFC in normal peripheral blood

Indicator	Rosette-forming cells, %	
	mean	range
BC_{gp}-RFC (n = 33)	11	6–21
Controls		
B_{ina}[1]-RFC (n = 22)	1.36	0–4
B_{PBS}[2]-RFC (n = 22)	0.57	0–4

[1] B incubated with heat-inactivated serum.
[2] B incubated with PBS.

Results

C3 binding to bacteria After incubation with fresh normal human or guinea pig sera up to a dilution of 1 32, $C3_h$ or $C3_{gp}$ was found to be bound to *E coli* as well as to *Salmonella*. No binding of C3 could be detected, when *E coli* had been incubated with PBS, heat inactivated sera or with C_h EGTA, C_h EDTA or C_{hu} ABS

Demonstration of BC_{gp} RFC in normal peripheral blood (table I) Peripheral blood lymphocytes of 33 individuals were checked for rosette formation with BC_{gp}. It was found that 6–21% (mean 11%) were BC_{gp} RFC. 22 control experiments were performed. After the incubation of B with heat inactivated sera 0–4% (mean 1.36%) and after the incubation of B with PBS 0–4% (mean 0.57%) of lymphocytes formed rosettes with B With increasing serum dilutions the number of BC_{gp} RFC decreased (correlation coefficient r = 0 94 $p<0\ 001$) A dilution of 1 64 showed no more rosettes.

Comparison of BC_{gp} RFC with EAC RFC In a series of 22 separately performed experiments the number of lymphocytes forming rosettes with EAC ranged from 5 to 30% (mean 12.63%) and the percentage of lymphocytes forming rosettes with BC_{gp} ranged from 6 to 21% (mean 12.95%) (correlation coefficient r = 0 64 $p<0\ 01$)

Demonstration of lymphocytes with membrane-bound immunoglobulins The percentage of lymphocytes with membrane-bound immunoglobulins ranged from 8 to 38% (mean 20.54%) (series of 14 experiments)

Combined assay for M Ig-Ly and BC_{gp} RFC (table II) In a separately performed combined assay for M Ig-Ly and BC_{gp} RFC (33 ex

ticipated without combined assay by the higher percentage of M-Ig cells (20.54%) in comparison to CRL cells (11%), and on the basis of this observation one might speculate that CRL are a subpopulation of M-Ig B lymphocytes. An alternative explanation might be however that a part of the M Ig$^+$-positive cells exhibit only passively absorbed Ig and are no real M Ig synthesizing B cells as recently reported by Lobo *et al.* [12] and Winchester *et al.* [18] while this study was performed. This will be investigated in further experiments. Our findings that about 50% of M Ig-positive and/or CRL-positive lymphocytes belong to the fraction M-Ig –CRL are comparable with the results of Ross *et al.* [16] who found about 60%, whereas Holm *et al.* [7] and Abrahamsson *et al.* [1] reported lower percentages (24 and 5% respectively).

As to the fraction M-Ig$^-$–BC$^+$ our results that about 20% are represented by this group are in agreement with Holm *et al.* [7] (30%). Abrahamsson *et al.* [1] and Ross *et al.* [16] on the other hand found 0 and 1% respectively. As to the actual classification of this group several explanations may be offered. This fraction might belong to the T cell subpopulation which is suggested by Chiao *et al.* [4] who observed lymphocytes bearing both C3 receptors and receptors for untreated sheep red blood cells, or to a subpopulation of lymphocytes characterized by the presence of Fc and C3 receptors but absence of Ig receptors and active in the lymphocyte-mediated lysis of antibody-coated target cells [9] or might be non-phagocytic monocytes with lymphocyte morphology. As far as our results show the subpopulation of lymphocytes with complement receptor sites seems to overlap with, but to be not totally identical with the subpopulation bearing membrane-bound Ig. This should be considered whenever these markers are used for the characterization of lymphocyte populations in lymphoproliferative and other diseases.

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incubation of *E. coli* with EGTA sera no binding of C3 to bacteria could be observed one may conclude that the binding of C3 from normal sera to *E. coli* is mainly due to the activation of C3 via the classical pathway. This indication is also supported by the fact that after incubation of *E. coli* with normal human sera previously absorbed with *E. coli* in the cold no C3 binding to bacteria could be demonstrated. Antibody against Enterobacteriaceae can be found in the sera of different species [17]. It is therefore suggestive that under our test conditions these antibodies are at least primarily responsible for the binding of C3 to bacteria. These natural antibodies seem to be non-IgG since bacteria incubated with heat-inactivated sera did not bind to Fc receptor lymphocytes.

Bacteria as indicator particles have several advantages over erythrocytes. They are not lysed after incubation with normal fresh sera; titration experiments for the estimation of the optimal antiserum and complement dilution can therefore be omitted. Monocytes can be easier recognized since bacteria are smaller than erythrocytes and allow a better judgement of the morphology of the central cell. The differentiation between lymphocytes and monocytes is further facilitated by the fact that most of the monocytes engulf complement-coated bacteria. In normal peripheral human blood we found 6–21% (mean 11%) of lymphocytes to form rosettes with BC_{rp}. A number of investigators reported similar percentages [1] using EAC as indicator cells while others [7, 14] found higher percentages (20–30%).

Our results of the simultaneous determination of BC_{rp} RFC and M Ig-positive lymphocytes showed that four different fractions could be detected: 2–20% (mean 8.33%) of the lymphocytes had both markers, 4–30% (mean 13.48%) carried only M Ig, but had no C3 receptors, 2–20% (mean 6.61%) had only complement receptors and 55–82% (mean 71.09%) had none of the two markers. Using a combined assay for the detection of EAC RFC and M Ig-positive lymphocytes in human peripheral blood, HOLM *et al.* [7], ROSS *et al.* [16] and ABRAHAMSSON *et al.* [1] could also detect four different lymphocyte fractions. As to the group M Ig$^-$$BC_{rp}$ our findings are in agreement with those of ROSS *et al.* [16] and HOLM *et al.* [7] who reported that 30–40% of the total of lymphocytes with complement receptor sites and/or membrane bound Ig is represented by this group, whereas ABRAHAMSSON *et al.* [1] found that 95% of lymphocytes with complement receptor sites and/or M Ig are M Ig$^-$EAC$^+$. The existence of a M Ig$^-$CRL group can already be an

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Histogenesis of Myeloid Metaplasia in the Spleen

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Key Words. Extramedullary hemopoiesis · Hemolytic agents · Myeloid metaplasia · RE cells · Splenic hemopoiesis

Abstract. Hemopoietic activity in the spleen is caused by the proliferation of the RE cells of the red pulp, lining the sinusoids and the endothelial cells lining the venous channels and their vasa vasora. The RE cells of the marginal zone do not contribute. As the cells involved line blood spaces, and the immature cells are washed into the circulation hemopoiesis appears to be intravascular.

Different agents produce different effects: copper sulphate an early proliferation of both the red pulp and venous cells, phenylhydrazine – red pulp proliferation and antiserum a high venous endothelial activity.

The role of the spleen in hemopoiesis is well known. From the studies conducted in this laboratory and in others [1] the exact site of origin of the hemopoietic cells within the spleen, and the cells of origin are still in dispute. In the present study an attempt has been made to ascertain the sites of involvement in this process. The effect of different agents has also been studied.

Materials and Methods

Hemopoietic activity was stimulated in the spleen by producing acute hemolysis. Three groups of 50 rats each were administered copper sulphate (5,000 μg/200 g b.w.), phenylhydrazine (20 mg/200 g b.w.), and anti-rat erythrocyte serum (1 ml of 1/10 dil/200 g b.w.), to produce approximately equal hemolysis after 24 h (i.e. 3 mg% serum bilirubin). These animals were sacrificed in batches of 10 each at intervals of 24 h, 48 h, 72 h, 5 days and 7 days. The spleen was removed and

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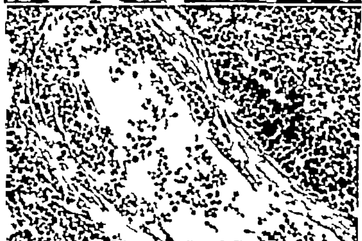


Fig 1 Dilated and congested sinusoids, 72 h after phenylhydrazine. The single layer of endothelial cells have become plump and protrude into the lumen. HE ×400.

Fig 2 Vein showing layers of proliferating cells arising from the endothelial cells. Many cells are seen to be loosened and washed into the lumen, 5 days after copper sulphate. HE ×100.

in 10% buffered formalin 5 m thick paraffin sections
... xylin and eosin (HE) Giemsa's stain, Wilders reticulin
... nin. The different zones in the spleen were studied in de-
... s were studied for the normal morphology

Results

...as observed that the arterioles within the Malphigian corpuscles, ... cuffing lymphocytes the marginal zone reticulo-endothelial (RE) cells and the germinal follicles did not show any hemopoietic activity The red pulp and the venous channels showed the following changes which followed a similar sequence with all three agents but with slight variations as will be shown later

Red pulp (fig. 1) At the commencement of the hemopoietic activity that RE cells lining the sinuses become plump with prominent vesicular nuclei Mitoses are seen within these cells. Clones of blast cells are formed with occasional megakaryocytes. These cells fan out from the central sinusoid. The cells gradually get converted into mature cells. At this stage the cohesion between the cells is lost and the cells are swept into the lumina Gradually the entire red pulp RE cells show activity

Veins (fig. 2) The venous endothelial cells also participate in the hemopoietic activity Along the lumen there are small projections produced by the proliferated endothelial cells These cells accumulate in the subendothelial zone They appear similar to the plump stem cells of the red pulp These cells gradually mature into erythroid and myeloid cells and lose their cohesion to be swept into the venous lumen The vasa vasorum of the venous channels become dilated Here and there hemopoietic cells are seen within these vessels by the proliferation of the lining endothelial cells The red pulp cells spill directly into the venous lumina through some of the prominent vasa which open from the red pulp directly into the lumen of the vein

The different hemolytic agents produced slightly different patterns of hemopoietic activity

Copper sulphate The proliferative activity of the red pulp commences within 24 h. The area of involvement gradually increases until by the 7th day the entire red pulp shows complete replacement by blood cells. The venous endothelium shows a similar activity Hemopoietic cells are seen to enter the lumina from the red pulp 2 days after administration

poietic activity also varies. Thus copper sulphate causes proliferation of the RE cells within 24 h. Both the red pulp sinusoidal cells and the venous endothelial cells are affected. These activated cells give rise to the hemopoietic cells. This process increases to a peak on the 7th day being enhanced by the hemolytic episode

With phenylhydrazine and antiserum there is no direct effect on the proliferation of the RE cells. This is evidenced by the lack of activity after 24 h. It is also seen that these two agents produce a marked dilatation of the sinusoids so that their outlines can be easily made out. With phenylhydrazine these sinusoids are filled with agglutinated masses of red cells. The lining RE cells appear stretched over these masses. Proliferative activity increases after 48 h and is seen to be more prominent after the 3rd day as compared to copper sulphate. This is in keeping with the progressive hemolysis taking place [6] The venous endothelium is less affected although it plays an important part in the activity

Antiserum at first causes destruction of the RE cells, the surviving cells being extremely inactive These cells start their activity after 48 h, the rise being very gradual up to the 5th day From the 5th to the 7th day there is a spurt in activity As compared to the other agents antiserum produces a prominent venous endothelial activity perhaps to compensate for the inactive red pulp. This activity remains high up to the 5th day On the 7th day there is a slight fall.

These observations show that the hemolytic agent is of importance in the production of hemopoietic activity Copper which produces a direct effect on the cells converts the RE cells into actively dividing cells. *Phenylhydrazine* and antiserum produce their effects mainly due to the hemolytic process and the feedback of red blood cell destruction. Antiserum shows a lag as there is a destruction of the red pulp RE cells at the outset.

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There is marked dilatation and congestion of the ... hese are filled with intact red cells. The sinusoidal ... tened and inert, stretched over the dilated space. ... ppears intact Due to the stretching the RE cells ... cut separately They become plump and active after 48 h ... nto the lumen From these cells groups of hemopoietic ... ll up the entire red pulp within 5 days This activity is ... that of copper sulphate The activity within the venous ... is less than that following copper sulphate, after 24 h. Later ... a rapid increase to a plateau after 3 days.

Intrv rum After 24 h there is a marked dilatation of the sinusoids which are filled with eosinophilic structureless material The lining cells of the sinusoids are destroyed in many places, so that some sinusoids are surrounded only by the reticulin framework. The few scattered surviving RE cells get activated after 72 h to replace the lost cells. These cells then continue to proliferate to form islands of hemopoiesis reaching a peak activity on the 7th day As compared to the other two groups venous proliferation is very much more prominent and commences within 24 h There is a rapid rise in activity reaching a peak on the 5th day

Discussion

From the above experimental results it is evident that although different hemolytic agents do have different properties the main sources of hemopoietic cells in the spleen in all cases are the RE cells lining the sinuses within the red pulp the venous endothelial cells and the vasa vasora Although the marginal zone RE cells proliferate particularly after copper sulphate [3] they do not participate in the hemopoietic activity The lymphoid cells of the Malphigian corpuscles are also not contributory although there is a prominent blast transformation with germinal follicle formation [5]

The proliferating cells line the sinusoids They are thus within the walls of the sinusoids and are just washed into the circulation as they mature. Thus it appears that intravascular hemopoiesis is the rule The hemopoietic cells enter the venous channels from the red pulp just as malignant cells do [4] by entering the dilated vasa which open directly into the lumen.

Different hemolytic agents produce different effects and the hemo-

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Circulating Platelet Aggregates and Thrombocytopenia Induced by Intravenous Infusions of Arachidonic and Lauric Acids in Guinea Pigs

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Key Words. Arachidonic acid · Lauric acid · Platelet aggregation · Thrombopenia · Thrombosis

Abstract. Slow intravenous infusion of Na laurate (NaL) into guinea pigs caused rapid appearance of platelet aggregates in the arterial blood and a precipitous fall in platelet counts. During the infusion of Na arachidonate (NaA) thrombocytopenia developed slowly, and few and smaller platelet aggregates appeared in the arterial blood. Considerably more guinea pigs died during or after the NaA infusion than after administration of NaL. The possibility that arachidonic acid and other long-chain fatty acids may play a role in the development of thrombosis and thromboembolism is discussed.

During prostaglandin synthesis from arachidonate endoperoxide intermediates (PGG_2 and PGH_2) are formed [6, 19, 24]. These substances are extremely potent initiators of platelet aggregation, and may play an important role in the causation of thrombosis and thromboembolism. Two recently reported model experiments lend support to this hypothesis. (1) Intravenous (iv) injections of small quantities of Na arachidonate (NaA) into rabbits caused accumulation of platelet aggregates in heart blood and pulmonary microcirculation leading to rapid death of the animals [16]. (2) The injection of the same fatty acid into the carotid artery of rats produced a stroke syndrome which was due to obstruction of the cerebral microcirculation by platelet aggregates [3].

Thromboembolism also occurs after iv injection of salts of many other long-chain fatty acids [2, 20, 25, 26]. The question was raised whether NaA caused experimental thrombosis by a specific biochemical mechanism (i.e. endoperoxide formation) or merely by a non-specific detergent

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action [22] This problem is of considerable practical interest because the above mentioned animal models are being used for the evaluation of potential antithrombotic drugs [4 17]

The experiments described in this paper were undertaken in an effort to find possible differences in the thrombogenic effect of NaA and the Na salt of another fatty acid lauric acid (NaL) The two substances were infused slowly iv into guinea pigs. It was thus possible to observe the development of platelet aggregates *in vivo* and their sequestration in the pulmonary microcirculation.

Materials and Methods

Guinea pigs of mixed breed and both sexes weighing from 530 to 810 g were used. The animals were anesthetized polyethylene catheters were implanted in the right jugular vein and the left carotid artery as described previously [28] Saline (0.9°/) was infused iv at a rate of 0 03 ml/h, using a compact Infusion Pump, Model 975 (Harvard App Co Millis, Mass.) After taking the first blood sample from the carotid artery a 0.2°/ (w/v) solution of NaA or a 0.5 / (w/v) solution of NaL were infused iv Injection speed was adjusted to provide 1 5, and 10 mg/kg/h of NaA and 5 10, and 20 mg/kg/h of NaL. Control animals received 5 ml/h of 0.9°/o saline. Blood samples were taken 10 20, 40, and 60 min after begin of the infusion After taking each sample the arterial catheter was carefully rinsed with 0.3 ml saline and closed with a metal cap After 1 h all animals received 0.9°/o saline iv for another 60 min at a rate of 5 ml/h They were then killed with an overdose of Na barbiturate iv

Circulating platelet aggregates were determined as follows a red cell pipette was filled to the 0.25 mark with Dilusol (Merz & Dade AG Bern) containing 1 /o (w/v) EDTA Na_2. The dilution fluid was slightly coloured with brilliant cresyl blue After mixing the pipettes on an electric vibrator for 5 min a Fuchs-Rosenthal counting chamber was filled. A total of 200 platelets were counted, and records of the number of platelets present as single cells or as aggregates (3 or more platelets) were made Two platelets adhering to each other were not counted as aggregate. Total platelet counts were determined with a Coulter Counter Model B using the method of RIEBER and STUDER [13] Counts obtained during the infusions were expressed as percent of the initial counts. In order to determine the reproducibility of the method multiple blood samples were evaluated in a few animals. Results summarized in table I indicate that the reproducibility is very good in samples containing few and small aggregates. The variability is higher in samples with more and larger aggregates.

Arachidonic acid, purity 99°/o, was a gift from Hoffmann-La Roche & Co AG Basle, Switzerland. 10-mg samples of the acid were dissolved in 1 ml 100°/ ethanol and stored under nitrogen in dark glass tubes at -20 °C Solutions for infusion were made fresh by mixing 1 part of the alcoholic solution with 4 parts of a 20°/ (w/) $NaCO_3$ solution. Lauric acid (purity 99.5 /) was purchased from Fluka AG Buchs,

Switzerland. A clear 0.5% (w/v) solution was obtained by addition of N OH until pH of 8.5–9.5 was reached.

Results

NaA caused death in the majority of the animals, most frequently during the 60 min following the fatty acid infusion. Only 4 of 15 guinea pigs receiving NaL and none of the 21 controls died (table II). The effect on platelets is demonstrated in figure 1. The infusion of 20 and 10 mg/kg/h of NaL caused a rapid drop in platelet counts which reached the lowest level after 10 and 20 min respectively. There was no further decrease despite continuation of the fatty acid administration. Significant numbers of platelet aggregates were demonstrated in the arterial blood after 10 min.

Table I. Multiple counting of platelet aggregates

Infusion	Time, min	Sample 1	Sample 2	Sample 3
0.9% NaCl	0	0	0	1.5
0.9% NaCl	40	1.5	3.5	
0.9% NaCl	60	4.5	3.5	5.0
0.9% NaCl	100	1.5	1.5	0
NaA 5 mg/kg/h	20	15.5	17.5	34
NaA 5 mg/kg/h	60	39	27.5	29.5

Table II. Lethal effects during and after intravenous infusions

Substance infused	Dose mg/kg/h	Number of animals treated	Number of animals dying during 60-min infusion	Number of animals dying after 60-min infusion
Na arachidonate	10	5		
Na arachidonate	5	8	0	6
Na arachidonate	1	5	1	3
Na laurate	20	5	0	1
Na laurate	10	5	1	0
Na laurate	5	5	0	2
0.9% saline	5 ml/h	21	0	0

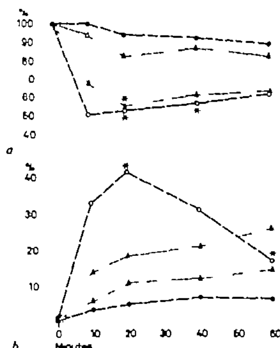


Fig 1 Platelet counts (a) and circulating platelet aggregates (b) during a 60 min iv infusion of NaL. ○ = 20 mg/kg/h ▲ = 10 mg/kg/h △ = 5 mg/kg/h ● = saline. Significantly different from saline controls ($p<0.05$ U test of Mann-Whitney).

With 20 mg/kg/h over 40% of the circulating platelets were present as aggregates in the blood sample taken after 20 min. After this the number of aggregates decreased but remained significantly elevated over the saline controls. With 10 mg/kg/h the percentage of platelets present as aggregates varied from 14 to 21% over the whole infusion period The majority of the circulating platelet aggregates were small Medium-size and larger aggregates containing up to 30 platelets were also observed particularly in the blood samples taken after 10 and 20 min The lowest dose of NaL (5 mg/kg/h) did not cause platelet aggregation and did not affect platelet counts.

During the infusion of 10 and 5 mg/kg/h of NaA platelet counts decreased slowly (fig 2) The counts were significantly lower than the controls after 10 min but the decrease continued as long as the NaA infusion was given Significant numbers of circulating platelet aggregates could not be detected in the arterial blood samples taken after 10 20 and 40 min After 60 min a slight increase in platelet aggregates was observed but it

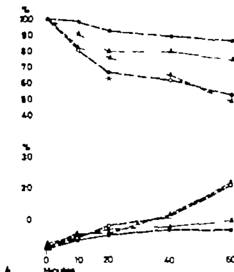


Fig. 2. Platelet counts (a) and circulating platelet aggregates (b) during 60 min iv infusion of NaA = 10 mg/kg/h ▲ = 5 mg/kg/h = 1 mg/kg/h ● = saline. Significantly different from saline controls (p 0.05, U test of Mann-Whitney).

was statistically different from the controls for the 5 mg/kg/h dose only. Most of the platelet aggregates were small; medium-size and large aggregates were only exceptionally seen. The lowest dose of NaA (1 mg/kg/h) had no effect on platelets.

Discussion

Slow iv infusions of NaA and NaL caused a dose-dependent decrease in platelet counts and appearance of circulating platelet aggregates in the arterial blood of guinea pigs. The development of these changes was considerably different with the 2 fatty acids. NaL caused a precipitous fall in platelet counts reaching the lowest levels after 10–20 min; there was no further decrease despite continuation of the infusion. With NaA platelet counts decreased gradually as long as the fatty acid was infused. Circulating platelet aggregates were present in significant numbers during the whole infusion of NaL, and some of the aggregates were medium-size or large. During the first 40 min of the NaA infusion the percentage of circulating platelets present as aggregates was not elevated over that seen in

aline controls. Only at 60 min did some small aggregates appear in the arterial blood

NaA is known to initiate rapid platelet clumping *in vitro* even at very low concentration [15 23] It is probable that the iv infusion of NaA also caused a significant degree of platelet aggregation The aggregates did not appear in the arterial blood but were retained almost quantitatively in the microcirculation of the lungs This resulted in a gradually developing thrombocytopenia and caused death in the majority of the guinea pigs during or shortly after the iv infusion Many small medium-size and large platelet aggregates were induced by the infusion of NaL. But these were only partially sequestrated in the capillary bed of the lungs Many were able to pass through the filter of the microcirculation and appeared in the arterial blood. This indicates that the aggregates formed under the influence of NaL had a lesser tendency to stick to endothelial surface of the pulmonary vasculature This may be the reason why only few animals died following the NaL infusions

The literature contains additional observations which shed light on the different thrombogenic properties of arachidonic acid and other long chain fatty acids. first of all many long-chain fatty acids do not cause rapid platelet aggregation *in vitro* as seen with NaA [15] Other fatty acids cause *in vitro* aggregation but the effect develops more slowly and often only in the absence of serum proteins [8 11] Platelets incubated with NaA released rabbit aorta contracting substances, but no release occurred with linoleic, linolenic, and stearic acid [23] *In vivo* five fatty acids structurally related to arachidonic acid did not kill rabbits when injected iv at doses 4 times higher than the LD_{50} of NaA [16] These differences between the biological effects of arachidonic acid and other long-chain fatty acids are probably a result of a diversity of their biochemical mechanisms. Long-chain fatty acids interact with platelet membranes making the platelets more sensitive to adenosine diphosphate induced aggregation [9] Aggregation occurring after such an interaction appears to be mediated through platelet adenosine diphosphate [7] and may thus be at least partly reversible. Arachidonic acid could of course, act in the same manner but it represents also an unique substrate of a prostaglandin synthetase enzyme complex [24] which is intimately linked with the process of platelet aggregation [18] If arachidonic acid is made available to the platelets, irreversible aggregation occurs promptly [15] Simultaneously inflammatory substances are released, an effect which does not take place with other fatty acids [23]

Our *in vivo* studies showed that aggregates formed during NaA infusion have a pronounced tendency to adhere to vascular endothelium and to obstruct the microcirculation. They are thus more likely to kill the animals than aggregates induced by NaL that are able to pass through the capillary system of the lungs. From the various model experiments with arachidonic and other long-chain fatty acids it appears conceivable that these substances play a role in the development of thromboembolic complications in man. It must be remembered, however that fatty acids are normally bound tightly to serum proteins or are esterified in platelet phospholipids [5 12, 21] They are released from their binding and storage sites through various physiologic and pharmacologic influences [1 10] and may then interact with platelet membranes. Intravenous infusions of fatty acid soap solutions, however represent only a crude approximation of the situation which might occur under conditions of endogenous fatty acid release. The model experiments reported in this paper as well as other demonstrations of fatty-induced thrombosis should therefore not be overrated. They demonstrate that the hemostatic equilibrium may be destabilized by naturally occurring fatty acids. This process can, under special experimental conditions, lead to widespread and even lethal thrombosis. It is not determined, however which role these mechanisms play in the development of various forms of human thrombosis and thromboembolism. The fact that non-steroidal anti-inflammatory drugs inhibit the thrombogenic action of arachidonic acid [16] and, less consistently also that of other long-chain fatty acids [14 27] will help in the evaluation of the clinical significance of these endogenous thrombogenic agents.

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Transferrin Immune Complex Disease

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Key Words: Atransferrinemia · Autoimmune diseases · Hemochromatosis · Immune complex disease · Iron metabolism · Iron overload · Transferrin antibody

Abstract. A 71-year-old woman showed a highly unusual pattern of iron distribution in the organism which was associated with iron overload. The hallmark of this disease was an extreme hypersiderinemia, the serum iron reaching about 800 μg/100 ml. There was a pigment cirrhosis of the liver, bronzed skin containing hemosiderin, and diabetes mellitus. Paradoxically, hemosiderin was not detectable in bone marrow macrophages, sideroblasts and erythrocytes were reduced, and there was a decrease in radioiron utilization of erythropoiesis, thus indicating insufficient iron supply. The pathogenesis of this disorder based on the formation of an autoantibody with specificity for transferrin thus producing a circulating immune complex which bound the majority of serum iron. Immunosuppression achieved a partial remission including a recovery of the patient's general state, a rise in free transferrin, a decrease in serum iron, disappearance of hemosiderin in the liver and a rise in erythrocyte production.

A hitherto unknown disorder of the iron metabolism was observed whose pathogenesis was attributed to the presence of an autoantibody associating with transferrin. Results of the protein analysis have been reported elsewhere [6–8]. The present paper describes the course of the disease and the variation of iron kinetics during immunosuppressive therapy.

Case Report

A 71-year-old woman attracted special attention by excessively high serum iron levels reaching about 800 μg/100 ml. The liver was slightly enlarged, indurated, and showed by laparoscopy the picture of a pigment cirrhosis. The histologic examina-

Table I

	Patient before therapy	during therapy	Normal values
Plasma iron binding			
Serum iron, μg/100 ml	780	408	50–150
Total iron-binding capacity μg/100 ml	812	507	270–370
Latent iron-binding capacity μg/100 ml	32	99	180–250
Transferrin-bound iron, %	10	42	95
Immune complex-bound iron, %	67	57	0
Micromolecular-bound iron, %	19	1	3
Ferrokinetics			
Intravascular T ½ of ^{59}Fe, min	540	165	70–120
Plasma iron turnover μg/h/100 ml blood	36	62	20–40
Bone marrow transit time of ^{59}Fe, days	8	5	3–4
^{59}F utilization by erythropoiesis, %	57	61	70–90
Erythrocytes			
Red cell mass, mg/kg	23	34	25–30
Reticulocyte count 10⁴/μl blood	6	13	4–8
T ½ of ⁵¹Cr-labeled erythrocytes, days		18	23–30

Determined by gel chromatography of the serum.

tion of liver biopsy revealed a microlobular cirrhosis with abundant amounts f hemosiderin in both hepatocytes and Kupffer cells. Diabetes mellitus and grayish colored skin containing hemosiderin deposits further indicated a severe iron over load of the organism. Paradoxically the iron overload was associated with several symptoms indicating deficient iron supply of erythropoiesis. These included an increased number of erythroblasts (40% of the myelogram), reduced number of sideroblasts (18%), a nearly complete absence of hemosiderin in medullar macrophages, moderate normochromic anemia (table I), and rise in urinary excretion of protoporphyrin (45 μg/100 ml) Prussian blue staining failed to detect hemosiderin in the duodenal mucosa.

Immune comple Serum protein analysis has been previously described in detail [6–8] I short, serum electropherogram showed an M-gradient in the β-region. In immunoelectrophoresis the transferrin band was intensified. Sephadex G-200 chromatography revealed an atypical brownish protein fraction containing 67% of radioiron added to the serum (tabl I). This protein had sedimentation coefficient of 9 9 S and dissociated at pH 3.5 into IgG and transferrin. At pH 7.6 the IgG component recombined with transferrin both from the patient and from normal individu-

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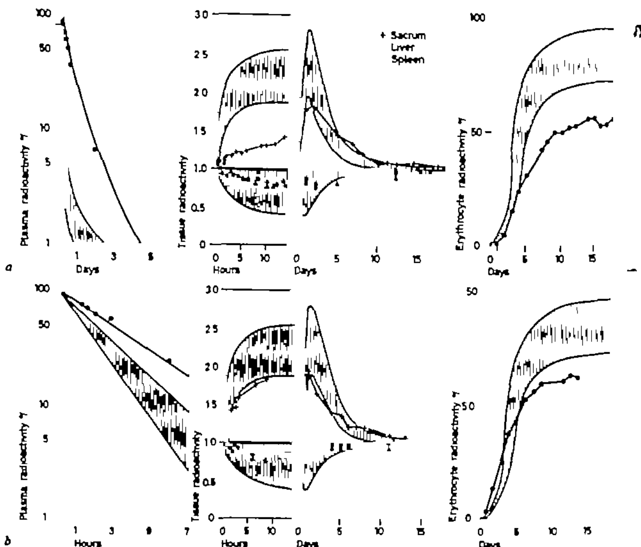


Fig 1 Ferrokinetic studies before (a) and during (b) immunosuppressive therapy (hatched areas represent normal ranges).

als. The complex consisted of one molecular IgG and of two molecules transferrin. The IgG component proved monoclonal IgG κ The kappa chains displayed abnormally large molecular size (27,000–28,000 dalton) due to a longer polypeptide. It, therefore, was concluded that the patient was producing an autoantibody with transferrin specificity This autoantibody formed a circulating IgG-transferrin immune complex.

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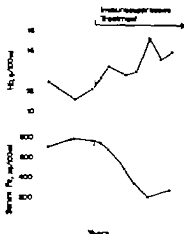


Fig. 2. Recovery from anemia and fall of serum iron during immunosuppressive therapy using azathioprine and prednisone.

540 min. Surface counting over the sacral marrow reflected delayed and reduced iron incorporation by erythroblasts. Bone marrow transit time of ^{59}Fe was prolonged to about 8 days, and ^{59}Fe utilization of erythropoiesis was reduced to 57%.

Immunosuppressive treatment. For 2 years azathioprine (100 mg/day) and prednisone (10 mg/day) were given continuously; later biweekly therapy phases with azathioprine (100 mg/day) and prednisone (25 mg day) were alternated with biweekly therapy-free intervals. During this therapy the patient's general state recovered considerably: the greyish skin color disappeared, and hemosiderin deposits in the liver could not be detected in a control biopsy performed after 1.5 years. The diabetes mellitus, however, persisted. Serum iron reduced from a maximum of 800 µg/100 ml to approximately 250 µg/100 ml (fig. 2). Hemoglobin normalized, temporarily even exceeding the normal levels (fig. 2). The fraction of iron bound to the immune complex decreased in favor of transferrin-bound iron (table I). The intravascular ^{59}Fe half-time reduced from 540 to 165 min, bone marrow transit time of ^{59}Fe shortened from 8 to 5 days, and ^{59}Fe utilization slightly increased from 57 to 61 (table I, fig. 1).

Discussion

The patient displayed simultaneously iron overload of several organs, extreme hypersiderinemia, and a deficient supply of erythropoiesis. This disorder arised from a disturbed iron transport due to the presence of an autoantibody with specificity for transferrin. By association with transfer

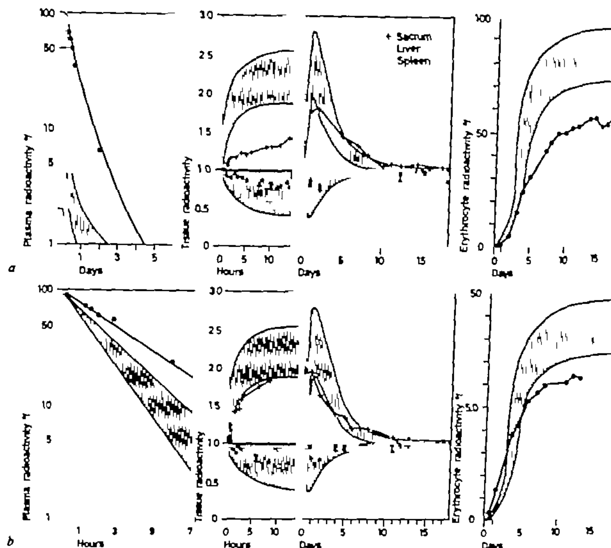


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rin it formed a circulating immune complex which contained the majority of serum iron

Immunosuppression achieved partial remission including a fall of serum iron a rise in free transferrin at the expense of the immune complex, disappearance of hemosiderin deposits in liver skin and recovery from anemia We, therefore may conclude that the immune complex caused an enhanced iron absorption with consecutive iron overload. However it did not guarantee sufficient iron supply to erythroblasts The high concentration of the immune complex in circulation and the extremely long intravascular half time of radioiron amounting to 540 min indicated a low clearance rate of this immune complex

HEILMEYER *et al* [4] described a case of congenital atransferrinemia displaying severe iron overload iron deficiency anemia, an almost complete lack of transferrin and an M-gradient in the γ-fraction which bound all radioiron added to the serum Interestingly transferrin emerged when mercaptopyridoxine a substrate which breaks S-S linkages, was added to the serum This observation equally indicated the presence of an immune complex. However in this case transferrin lost its detectability by anti transferrin due to its association with the antibody

Besides the assumed existence of an autoimmunoatransferrinemia GOYA *et al* [2] described the genuine atransferrinemia arising from an in born failure of transferrin synthesis. This form as well as an acquired type of atransferrinemia by renal transferrin loss in severe nephrosis [3] does not go along with significant iron overload.

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6 WERNET P KICKHÖFEN B WESTERHAUSEN M and SVEHAG, S. E. A mono-

Materials and Methods

Haematological examinations were carried out by standard methods [2]. Hb F percentages were measured using the SINGER method. The presence of thermolabile haemoglobin was demonstrated by the method of DACIE *et al.* [3] at 50 °C and by that of CARRELL and KAY [4] at 37 °C. Haemoglobin electrophoresis of haemolysates was carried out in cellulose acetat with Tris-EDTA buffer pH 9.1. The electrophoresis of globin was carried out in cellulose acetate using 6 M urea 0.025 M barbital buffer pH 8.

The abnormal haemoglobin was prepared by column chromatography on DEAE Sephadex according to the method of HUISMAN and DOZY [5]. The haemoglobin was converted to globin by acid/acetone precipitation at 0 °C (washing 3 times in acetone at 0 °C) [6] and the abnormal β-chains isolated as described by CLEGG *et al.* [7]. Diagnostic finger-prints of the tryptic digests of the globin chains were prepared and stained with ninhydrin (0.2% in acetone) and with reagents specific for methionine, histidine, arginine, tyrosine and tryptophan [8]. Preparative finger-prints (containing the digest from about 3.5 mg of globin each) were stained with 0.02% ninhydrin in acetone. Elution of the abnormal peptide in NH_4OH and digestion with thermolysin was carried out as described before [9]. For analyses, abnormal peptides were eluted in 6 N HCl, hydrolysed in sealed capillary tubes at 110 °C for 18 h, dried and their amino acid compositions determined on an automatic amino acid analyser.

For preparative purposes, it was found easier to prepare CARRELL and KAY [4] precipitate of this unstable haemoglobin, dissolve the precipitate in 0.1 N HCl with the aid of some urea and prepare globin (as above). This whole globin was then digested and finger-printed to isolate the abnormal β-peptide (which separated well from other α- and non-α-tryptic peptides at pH 6.4).

Case History

The family is of Sicilian extraction (Caltanissetta). The parents are unrelated. The father has suffered from chronic haemolytic anaemia since childhood. The aetiology of the anaemia was never recognized. The mother had 5 pregnancies one of which produced twins. She has 3 sons and 3 daughters (fig. 1). The 3 daughters were admitted to the Paediatric Clinics of Pavia (August 1974) for more careful investigations:

A. Maria was born in 1964. When she was 20 months old, she suffered from haemolytic crises associated with a fever episode. She received 10 blood transfusions at different times. At the clinical examination, only a slight splenomegaly was observed.

Rosa was born in 1967. When she was 30 months old, she had a fever haemolytic crisis and splenomegaly. She received 6 blood transfusions. At the clinical examination, slight scleral jaundice and moderate splenomegaly were found.

Patrizia was born in 1970. When she was 20 months old, fever and haemolysis

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Haemoglobin Shepherds Bush (β74 [E18] Gly→Asp) in an Italian Family

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Key Words. Haemoglobin Shepherds Bush   Haemoglobinopathies   Unstable haemoglobins

Abstract Haemoglobin Shepherds Bush has been found once before in a woman of British origin. It is an unstable haemoglobin with a raised oxygen affinity This paper reports its discovery in a father and 3 of his daughters in Sicily

Examination of the blood samples from 3 daughters of a Sicilian family who had been admitted into hospital during a fever and haemolytic crisis, showed the presence of an increased haemoglobin A_2 fraction and an abnormal haemoglobin moving slightly more anodically than haemoglobin A on cellulose acetate at pH 9 1 The heat instability test was positive indicating an unstable haemoglobin and Heinz bodies were found in the red cells. The unstable haemoglobin was haemoglobin Shepherds Bush.

Haemoglobin Shepherds Bush has been described only once before, in a 30-year-old South African woman of British origin [1] This variant was found to be an unstable haemoglobin and was associated with chronic anaemia in childhood before removal of the spleen The substitution in the β-chain of the haemoglobin represents an instance where a charged amino acid (aspartic acid) is introduced into the haem pocket, the consequences of which are to weaken the hydrophobic forces which prevent access of water to the haem pocket.

Table I. Haematological values of the 3 daughters of the family

	A. Maria	Rosa	Patrizia
Haemoglobin, g/100 ml	13.5	12.2	13.3
RBC, $10^6/\mu l$	4.2	3.5	4.1
MCV, µm	89	94	85
MCH, pg	31.2	34.5	31.7
MCHC, %	35.4	36	36.7
Reticulocytes, %	12	8	7
Osmotic fragility test	normal	normal	normal
Serum bilirubin, mg/100 ml			
Total	1.89	3.15	1.80
Direct fraction	0.60	1.08	0.84
G6PD mU 10	59		182
Serum iron, µg/100 ml	82	125	116

Table II. The amino acid composition (molar ratios) of βTpIX (β67–82) from haemoglobin Shepherds Bush

Amino acid	Haemoglobin Shepherds Bush βTpIX	Expected in haemoglobin A βTpIX
Asp	3.88	3
Ser	0.95	1
Gly	1.12	2
Ala	2.12	2
Val	0.93	1
Leu	3.97	4
Phe	1.10	1
His	0.94	1
Lys	0.99	1
Yield residue, nmol	9.6	

missing from their usual position in this abnormal haemoglobin. Instead, a new spot which gave a positive reaction for histidine was found close to the position of βTpV + βTpXIIIa. The amino acid analysis of this peptide is given in table II and was found to be identical with that expected from βTpIX except that one residue of glycine was missing and had been replaced by one residue of aspartic acid. The assumed position of the new

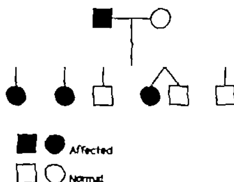


Fig 1 Family tree of the Sicilian family

occurred, associated with splenomegaly She received 2 blood transfusions. At the clinical examination, marked hepatosplenomegaly was found to be present.

Results

Many routine examinations were carried out on the 3 daughters (table I) Coombs test Ham test sickling test and serum transaminases were all found to be normal.

The lability tests were positive after only a few minutes in all the patients, including the father by both the DACIE *et al* [3] and the CARRELL and KAY [4] methods. The 4 haemolysates contained normal amounts of Hb F The electrophoretic patterns on cellulose acetate at pH 9 1 showed an increased haemoglobin A_2 fraction After a few days of storage of the haemolysates at –20 C, a haemoglobin fraction appeared moving more anodically than Hb A but incompletely separated from Hb A

Cellulose acetate electrophoresis of total globin using 6 M urea 0 025 M barbital buffer pH 6 8 showed an abnormal β-chain more negatively charged than normal β-chain. The chain separation of globin prepared from a 5-min isopropanol precipitate showed normal α-chains and only abnormal β-chains. When the blood of the 3 daughters was incubated with brilliant cresyl blue, a large number of red cells showed inclusions exactly identical to that of Hb H disease.

The finger print of the soluble tryptic peptides from whole globin prepared from a CARRELL and KAY [4] precipitate is shown in figure 2 When compared with the finger-print of the soluble tryptic peptides obtained from a haemoglobin A βTpIX and βTpVIII IX were observed to be

Table III The amino acid composition (molar ratios) of the thermolysin peptides β71–74 and β71–75 from haemoglobin Shepherds Bush

Amino acid	Haemoglobin Shepherds Bush		Expected in haemoglobin A	
	β71 74	β71 75	β71 74	β71 74
Asp	2.04	2.07	1	1
Ser	0.96	0.94	1	1
Gly			1	1
Leu		0.99		1
Phe	0.65	0.62	1	1
Yield per residue, nmol	19.9	8.1		

N-terminal Phe destroyed during acid hydrolysis

en in table III and were found to correspond to residues 71–74 and 71–75 except that in each case the residue of glycine was missing and an extra aspartic acid was found

Since this abnormal haemoglobin has a charge difference from normal haemoglobin (as seen on cellulose acetate, paper and starch-gel electrophoresis at alkaline pH, and on globin-chain separation on carboxymethyl-cellulose chromatography at pH 6.7) and since the new peptides βTpIX, residues 71–74 and residues 71–75 were more negatively charged than usual, the substitution was assumed to be to aspartic acid rather than asparagine. Hence the new haemoglobin was haemoglobin Shepherds Bush, β74 Gly→Asp.

Discussion

It is of interest that haemoglobin Shepherds Bush has been found in a Sicilian family where although causing illness, it seemed to be compatible with survival. In haemoglobin Shepherds Bush, the substitution of glycine 74 (E18) by aspartic acid is easily accommodated in the oxy-structure. However in the deoxy-conformation, the acidic side chain of the aspartic acid moves very close to phenylalanine 85 (F1) which makes the tertiary deoxy-structure unstable. This results in this abnormal haemoglobin having a raised oxygen affinity [10] The raised oxygen affinity of the blood of the heterozygotes would cause a compensatory stimulation of red cell

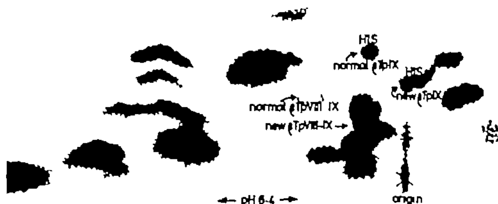


Fig 2 Haemoglobin Shepherds Bush. Finger-print of the soluble tryptic peptides from whole globin



Fig 3 Haemoglobin Shepherds Bush. Finger print of the peptides derived from thermolysin digestion of the abnormal βTpIX

βTpVIII IX was not noted on staining due to the presence of βTpXI at the same place, which also stained positively for histidine

The finger-print of the peptides derived from thermolysin digestion of the abnormal βTpIX is given in figure 3 The peptide, residues 71–74 was seen to be missing and two new peptides were observed which were more negatively charged The analyses of these two new peptides are giv

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Interaction of Haemoglobin E with α Thalassaemia and Haemoglobin Constant Spring[1]

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Key Words. α-Thalassaemia · Hb CoSp · Hb E · Haemoglobinopathies · Haemolytic anaemia

Abstract. The combination of Hb E, α-thalassaemia and Hb CoSp was found in a 20-year-old female Malay who presented with moderately severe haemolytic anaemia. The findings in the patient and her family from which this diagnosis was arrived at are discussed. Although this is the first report of this condition in this country it is pointed out that one may see more such cases in the future if one is aware of this condition since Hb E, α-thalassaemia and Hb CoSp all occur at significant frequencies in this country.

Haemoglobin H disease, a form of α-thalassaemia, is one of the common causes of hereditary haemolytic anaemia in this country [9]. According to current concepts on the genetic pattern of the α-thalassaemia syndromes, Hb H disease is due to a patient inheriting three α-thalassaemia genes, two of which are inherited from one parent, who has a severe α-thalassaemia trait, and one inherited from the other parent, who has a mild α-thalassaemia trait. Studies carried out at this Institute by Lie Injo *et al.* [7] showed that two types of Hb H disease exist, one type associated with the presence of Hb Constant Spring (Hb CoSp), at that time designated Hb X, which is invariably present in one parent and the other type not associated with the presence of Hb CoSp. The clinical and haematological features of patients with and without Hb CoSp have

[1] This work was supported in part by the University of California International Centre for Medical Research (UC ICMR) through research grant AI 10051, and in part by research grant HL 10486, both from the National Institutes of Health, US Public Health Service.

production and this may account for the almost normal haemoglobin level of the patients. In addition to the structural abnormality causing a shift to the oxy form, it has also been shown that haemoglobin Shepherds Bush has a reduced affinity for 2,3-diphosphoglycerate [11] This would also cause the haemoglobin in the presence of 2,3-diphosphoglycerate, to tend to exist in the oxy rather than the deoxy-conformation

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Interaction of Haemoglobin E with α-Thalassaemia and Haemoglobin Constant Spring[1]

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Key Words α-Thalassaemia Hb CoSp Hb E Haemoglobinopathies Haemolytic anaemia

Abstract The combination of Hb E, α-thalassaemia and Hb CoSp was found in 20-year-old female Malay who presented with moderately severe haemolytic anaemia. The findings in the patient and her family from which this diagnosis was arrived at are discussed Although this is the first report of this condition in this country it is pointed out that one may see more such cases in the future if one is aware of this condition since Hb E, α-thalassaemia and Hb CoSp all occur at significant frequencies in this country

Haemoglobin H disease, a form of α-thalassaemia, is one of the common causes of hereditary haemolytic anaemia in this country [9] According to current concepts on the genetic pattern of the α-thalassaemia syndromes, Hb H disease is due to a patient inheriting three α-thalassaemia genes, two of which are inherited from one parent, who has a severe α-thalassaemia trait, and one inherited from the other parent, who has a mild α-thalassaemia trait. Studies carried out at this Institute by Lie Injo *et al.* [7] showed that two types of Hb H disease exist. one type associated with the presence of Hb Constant Spring (Hb CoSp), at that time designated Hb X, which is invariably present in one parent and the other type not associated with the presence of Hb CoSp The clinical and haematological features of patients with and without Hb CoSp have

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been found to be essentially similar Evidence that the Hb H disease not associated with Hb CoSp is due to the inheritance of three α-thalassaemia genes is found in the work reported by KAN *et al* [3]

In Malaysia, Hb E is the most frequent abnormal haemoglobin [4 8] while α thalassaemia is also prevalent and Hb CoSp [2, 6] is found in appreciable frequencies One would therefore expect to find families in which these three genes interact. The following is a report of such a family

Methods

Haematological examinations were carried out according to standard methods [1] Haemolysates were prepared from washed packed red cells by the addition of one volume of water and 0.5 volume of toluene. Haemoglobin F levels were measured by the method of SINGER *et al* [11]. Electrophoresis of haemoglobin was done on starch gel using Tris-EDTA boric acid buffer at pH 8.6 and discontinuous Tris-boric acid buffer at pH 9.5 Cellulose acetate electrophoresis was done in Tris-EDTA boric acid buffer at pH 8.9 Haemoglobin A_2 and other haemoglobin components were quantitated by the cellulose acetate electrophoretic method of MARENGO-ROWE [10]

Findings in the Family

We have for some time been examining patients with Hb H disease and their families for clinical and haematological findings. The family study of one such Malay patient with Hb H disease with Hb CoSp led to the finding of the family that is being reported. Unfortunately in this family the father a Malay had died several years ago The mother also a Malay and her four children were examined. One of these children, a son, was the patient with haemoglobin H disease with Hb CoSp He was 16 years old at the time of examination and he gave a history of pallor and recurrent episodes of fever and jaundice for several years. When admitted to the District Hospital Kuala Pilah, during one such episode of fever and jaundice he was found to have hepatosplenomegaly and on haemoglobin analysis the diagnosis of Hb H disease with Hb CoSp was made. Subsequently all other living members of the family were examined Except for child 3 (table I) none had any complaints and were quite well Child 3, the proposita in this report, was a 20-year-old female who worked as a hospital 'amah She gave a history of pallor and recurrent episodes of fever for several years. On examination she was found to look pale and to have an enlarged spleen extending 1½ finger breadths below the coastal margin. X rays of the chest, skull and hands did not show any abnormal finding. The haematological findings in the proposita and the rest of the family are shown in table I The peripheral blood film of the proposita showed fairly marked hypochromia and marked anisopoikilocytosis with many target cells, microcytes, macrocytes and schistocytes and some spherocytes, elliptocytes and pear-shaped cells. Haemoglobin electrophoresis was done on cellulose

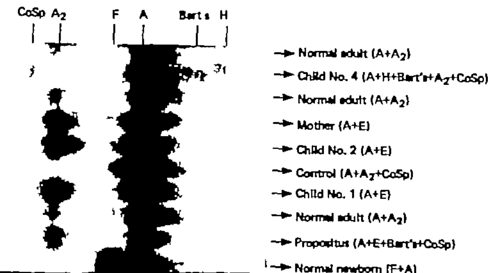


Fig 1 Starch gel electrophoresis in Tris-EDTA boric acid buffer pH 8.6 showing the haemoglobin patterns of the proposita and those of her mother brothers and sister compared with those of normal controls.

acetate and on starch gel at pH 8.6 and 9.5 Starch gel lectrophoretic patterns at pH 8.6 are shown in figure 1 The total whit cell count was 9 400/μl and the platelet count 270,000/ l The Motulsky test for G6PD deficiency was normal. From the above findings and the findings show in table I it can be seen that the proposita had moderately severe haemolytic anaemia similar in severity to that of her 16-year-old brother (child 4 in table I) who had Hb H disease with Hb CoSp a condition well known to cause haemolytic anaemia of this degree. But, unlike in the patient with Hb H disease with Hb CoSp (child 4) in child 3 (the proposita) w found H inclusions in only occasional red cells (1 in 1,000 or more red cells); these inclusions were similar to H inclusions but were a little larger and more uneven. Haemoglobin electrophoresis did not show Hb H but only haemoglobins A and E together with Hb CoSp and small amount of Hb Bart's (tabl I).

The mother in this family had heterozygous Hb E with the severe form of α-thalassaemia trait, the presence of the severe form of α-thalassaemia trait was show by the finding of H inclusions in about 1 in 4,000 red cells. Child 1 had condition identical to that of the mother And both of them did not have any anaemia but the red cells showed ery mild anisopoikilocytosis with few microcytes and elliptocytes. The level of Hb E particularly in the mother but also in child 1 was lower than the level of Hb E in child 2. Studies by Thai workers [12] ha show that the amount of Hb E in the heterozygous state is reduced in the presence of α-thalassaemia trait. Child 2 had the heterozygous state for Hb E.

Table I Haematological

	Age years	Hb g%	RBC × 10⁶/ μl	PCV %	MCV μm³	MCH pg	MCHC %	Reticulocytes %	RBC morphology	H' inclusions
Mother	50	11.8	5.0	36.8	73.2	[illegible]3.4	31.9	1.0	+	occasional
Sister (child 1)	23	12.1	4.6	35.5	76.8	26.2	34.1	0.4	±	occasional H' incl.
Brother (child 2)	22	14.[illegible]	5.9	43.0	72.9	24.1	33.0	1.0	0	nil
Proposita (child 3)	20	8.7	4.5	32.0	71.9	19.4	27.0	4.2	4+	occasional inclusions similar to H' incl.
Brother (child 4)	16	9.8	4.4	38.6	88.6	22.4	25.3	4.8	4+	+ + +

t = total d = direct I = indirect.

Discussion

Child 4 in this family had Hb H disease with Hb CoSp Apparently he had inherited the α-thalassaemia$_1$ trait from the mother and Hb CoSp from the father In addition to the α-thalassaemia trait the mother had Hb E which was not inherited by child 4 The finding of occasional red cell inclusions and decreased amount of Hb E in comparison with the level usually seen in Hb E trait carriers are in agreement with the idea that the mother had α-thalassaemia trait in addition to Hb E. The proposita (child 3) had inherited the α-thalassaemia trait as well as the Hb E trait from the mother and Hb CoSp apparently from the deceased father She therefore had the genes for both Hb E and Hb CoSp and at the same time had α-thalassaemia this combination had led to the haemolytic condition

ngs of the family

otic lity	Hb pattern	Hb F %	Hb A_2 %	Hb E %	Hb Bart's %	Hb H %	Hb Co-Sp %	Serum bili-rubin mg%	Se-rum iron µg%	TIBC µg%
to left	A+E	1.2		20.0				t 0.5 d 0.2 i 0.3	74	282
to left	A+E	0.8		27.4				t 0.5 d 0.3 i 0.2	85	282
	A+E	1.2		31.8				–	–	–
ked 1 to left	A+E+Bart's+CoSp	1.7		16.0	1.9		1.0	t 1.4 d 0.5 i 0.9		207
y slight t to left	A+H+Bart's+A_2+CoSp	1.8	2.5		Bart's+H 20.0		1.0	t 1.5 d 0.8 i 0.7	113	165

TUCHINDA *et al.* [12] and WASI *et al.* [13, 14] have reported several cases of a disorder thought to result from the interaction of Hb E with α-thalassaemia$_1$/α-thalassaemia$_2$. The patients reported by WASI *et al.* had clinical and haematological findings not different from those of Hb H disease. On haemoglobin electrophoresis in their patients, the major haemoglobins found were A+E+Bart's and the Hb E level was lower than that found in simple Hb E heterozygotes, as was also the case in our patient. WASI *et al.* reported that with both starch-gel and cellulose acetate electrophoresis a faint band of a slow-moving haemoglobin was seen cathodal to Hb E at pH 8.6 and that the amount of this slow-moving Hb was 0.53–1.30%. WASI *et al.* reported that these slow haemoglobin bands were similar to those observed in Hb H disease. Perhaps this slow-moving haemoglobin was in fact Hb CoSp. Familial findings in their cases were also similar to those in Hb H disease and in many in-

stances both diseases were divided among the siblings. One parent usually exhibited the characteristics of α-thalassaemia$_1$ trait and the other had normal haematological features and was presumably the carrier of the α-thalassaemia$_2$ trait. Hb E was found in at least one of the parents. WASI *et al* believed that these patients with haemoglobins A+E+Bart's carried the same genes as Hb H patients but inherited a Hb E gene in addition thus having the genotype of α-thalassaemia$_1$/α-thalassaemia$_2$-Hb E (Hb H/Hb E) The finding that newborns with haemoglobins A+F+E+25% Hb Bart's developed Hb A+E+Bart's disease whereas neonates with haemoglobins A+F+25% Hb Bart's developed Hb H disease was thought to be further evidence for the validity of their theory However we have shown [7] in numerous families, that α-thalassaemia in combination with Hb CoSp alone can lead to Hb H disease with the presence of Hb CoSp Apparently before we elucidated the significance and mode of inheritance of Hb CoSp, the Thai workers thought it to be the expression of α-thalassaemia$_2$ and they diagnosed the reported cases as combination of Hb E and α-thalassaemia$_1$/α-thalassaemia$_2$. At least part of their cases must have been combinations of Hb E, α-thalassaemia$_1$ and Hb CoSp as is the case in the patient reported in the present paper We have also shown [5] that Hb CoSp (HB X) in the newborn period is usually accompanied by Hb Bart's. The findings in the family of the proposita in this report were similar to those in the families of the patients reported by WASI *et al* [13 14] The mother had Hb E and α-thalassaemia$_1$ trait. The father had died before we examined this family But the fact that the proposita had Hb CoSp and a brother had Hb H disease with Hb CoSp shows that the father must have had Hb CoSp. With these findings in the family and the finding in the proposita of clinical and haematological features of a moderately severe haemolytic anaemia together with haemoglobins A+E+Bart's+CoSp and only occasional inclusions in the red cells, we believe that the proposita had the combination of Hb E, α-thalassaemia$_1$ and Hb CoSp. Since Hb E, α-thalassaemia and Hb CoSp all occur at significant frequencies in Malaysia it is not surprising to find the combination of Hb E, α-thalassaemia$_1$ and Hb CoSp in our population This is however the first report of this condition in this country It is important to be aware that this condition can cause a moderately severe haemolytic anaemia and to make the correct diagnosis when a patient is encountered. If one misses the diagnosis or wrongly diagnoses this condition as simple heterozygous Hb E the patient will be subjected to unnecessary repeated investigations to

determine the cause of the anaemia and in the mean time unwarranted and sometimes harmful amounts of iron therapy may be given by unwary clinicians in an effort to correct the anaemia.

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Red Cell Pyruvate Kinase Deficiency: Adverse Effect of Oral Contraceptives

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Key Words. Anovulant drugs · Contraception · Enzymerythropathies · Erythrocyte metabolism · Haemolytic anaemia · Pyruvate kinase deficiency

Abstract. Erythrocyte enzymopathies are being detected with increasing frequency. The homozygous state for pyruvate kinase (PK) deficiency usually causes chronic non-spherocytic haemolytic anaemia. Aggravation of the condition by a variety of extrinsic factors has been reported, but drugs have rarely been incriminated. A case of severe PK deficiency is presented in which an anovulant drug upsets the usual haematologic balance. This strengthens the suspicion that contraceptive pills may be hazardous in such patients.

Haemolytic anaemia due to deficiency of erythrocyte pyruvate kinase (PK) was first reported in 1961 by VALENTINE *et al.* [1]. Ten years later TANAKA and PAGLIA [2] provided an exhaustive review of the subject and noted that over 135 cases had by that time (1971) been identified. To date the count may well be close to 200. Of all the hereditary red cell enzyme deficiency states, PK deficiency is only exceeded in frequency by glucose-6-phosphate dehydrogenase (G6PD) deficiency. Although the condition is thought to affect northern Europeans predominantly, there is increasing evidence for world-wide distribution and as yet its incidence in many parts of the world is unknown. The degree of haemolysis and anaemia varies considerably in PK deficiency, some cases requiring frequent transfusions, others remaining asymptomatic for years.

Autosomal recessive inheritance of PK deficiency is generally accepted, the clinical condition being detectable almost exclusively in homozygotes or double heterozygotes. Usually there is no overlap of PK assay values between homozygotes and heterozygotes, the former ranging be-

tween 5 and 25% and the latter about 50% of the normal mean [2]. There may however be some overlap between the heterozygous range and the low normal. Interestingly there seems to be poor correlation between the PK value and the severity of the haemolytic process. This observation led to the suspicion, borne out in recent years [3, 4] that many cases of PK deficiency may be due to kinetically aberrant isozymes.

As in other hereditary haemolytic conditions, the anaemia in PK deficiency may be aggravated by stress factors such as infection and pregnancy [5, 6]. Drugs have not been incriminated as they have in other enzymic disorders such as G6PD deficiency. However, in at least one instance, an anovulant drug has been suspected of intensifying the haemolytic process [7]. Another case is now reported in which a contraceptive pill appears to have caused significant deterioration of the previously stable haematologic status of a patient with PK deficiency.

Report

A 35-year-old French Canadian female, whose parents were remotely related, gave a vague history of mild icterus since childhood. She experienced two uneventful pregnancies at the ages of 24 and 28 years. Towards the end of the first trimester of her third pregnancy, at the age of 33, she became symptomatic for the first time, and was found to have a haemolytic anaemia. This led to the diagnosis of severe erythrocyte PK deficiency. A therapeutic abortion was performed and 2 months later splenectomy and cholecystectomy were carried out. The gall-bladder contained numerous pigment calculi. Folic acid supplementation was taken regularly after the diagnosis of PK deficiency was established.

She was asymptomatic during the months following splenectomy, with haemoglobin and PCV levels remaining about 12 g/dl and 38%, respectively, reticulocytes 10%, and serum bilirubin about 3 mg/dl. Approximately 1 year after splenectomy she was placed on the anovulant drug Norinyl® 1/80 (Norinyl 1/80 [Syntex] = norethindrone 1 mg + mestranol 0.08 mg). The only other medication taken during this period was folic acid. During the 3rd month on the contraceptive pill she noted increasing malaise and fatigue. The haemoglobin level was found to be 9.5 g/dl, PCV 29% and reticulocytes 47%. Norinyl was discontinued and there followed a gradual decline of the reticulocytosis, rise in haemoglobin and PCV (fig. 1) and a return to her previous sense of well-being.

Special studies. The proposita, considered to be a homozygote for PK deficiency was studied during a stable period of her haemolytic process. Her two sons, obligatory heterozygotes, were also examined. Results are given in table I.

Red cell PK was measured by the method described by Beutler [8] in which the change in optical density caused by the oxidation of NADH is monitored. Leukocyte contamination was minimized by PVP sedimentation of the erythrocytes.

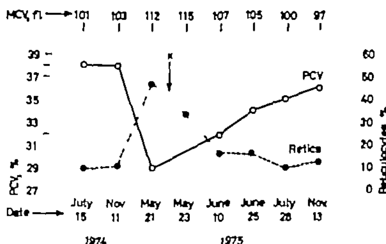


Fig 1 Haematologic values at irregular intervals over a 16-month period. Indicates time the drug was stopped.

Table 1 Family studies

Subject	Age, years	Hb g/dl	Reticulocytes %	Pyruvate kinase IU/g Hb
Proposita	35	12.2	13	0.74
Son (1)	11	1_.8	1 6	3.84
Son (2)	7	12.9	_.8	3.74
Normal values	–	–	<3	range 4 07 7.31

Results and Discussion

The proposita is able to maintain a normal haemoglobin level despite the severely reduced red cell PK activity the consequent block in the Embden Meyerhof glycolytic pathway and the shortened red cell life span. This is achieved by increased erythroid activity in her bone marrow The importance of splenectomy in this case cannot be stated since pre-splenectomy assessment was incomplete. Her erythrocytes show a severe deficiency of PK activity The two sons of the proposita show no haematologic abnormalities their erythrocyte PK levels are slightly below the normal range

Although they may be haematologically asymptomatic much of the time, individuals with severe PK deficiency are probably in a rather pre-

carious homeostatic equilibrium with respect to their red cells. The fact that an anovulant drug upsets this equilibrium in the present case attests to this probability. Although a cause and effect relationship with the drug could not be proven beyond doubt, the fact that this patient had only suffered one previous symptomatic haemolytic episode, and that while pregnant, lends strong support to the presumption. The mechanism by which contraceptive medicines might intensify a red cell enzyme defect is not clear. However, some 10 years ago a patient was reported by OSKI *et al.* [9] to be doubly heterozygous for G6PD and PK deficiency and to have a mild haemolytic condition. This raised the possibility that a mild enzyme deficiency in the pentose phosphate pathway could magnify the effects of a mild PK deficiency. More recently, MARKKANEN *et al.* [10] have reported a reduction in red cell transketolase activity in the pentose phosphate pathway in women during anticonceptive hormone therapy. This perhaps provides a clue as to how such therapy might interact unfavourably with the PK deficiency state.

Women with severe PK deficiency may tolerate pregnancy reasonably well, as did the present case on two occasions. However, the burden of pregnancy may have an untoward effect in some instances, leading to maternal and fetal morbidity. If such women decide or are advised against pregnancy, they should probably avoid the use of anovulant drugs.

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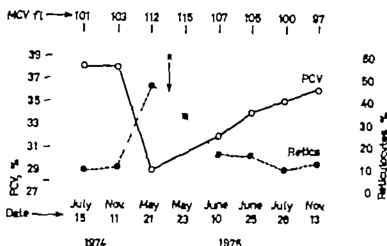


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Glucose-6-Phosphate Dehydrogenase Velletri

A New Variant with Reduced Activity in a Patient with Congenital Non-Spherocytic Haemolytic Anaemia

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Key Words. Congenital non-spherocytic haemolytic anaemia Enzyme erythropathy Erythrocyte metabolism G6PD Velletri Haemolytic anaemia Red cell G6PD

Abstract. A new variant of red cell glucose-6-phosphate dehydrogenase (G6PD) has been found in Caucasian man with congenital non-spherocytic haemolytic anaemia. This variant has reduced activity increased thermolability increased Michaelis constants for glucose-6-phosphate and NADP slightly increased electrophoretic mobility and biphasic pH-activity profile The red cell adenine compounds and ATP are in normal limits. The increased activity of red cell NADP-glutathione reductase is probably the expression of mechanism of compensation for the decrease of G6PD and consequence of the decrease of NADPH.

Studies on electrophoretic mobility on Michaelis constants for the specific substrate and for analogous substrates, on the heat stability and on the pH-activity profile, have made it possible to identify more than 80 principal variants of red cells glucose-6-phosphate dehydrogenase (G6PD) which correspond to X-linked mendelian characteristics [1 6] This paper describes a variant with reduced activity increased thermo-instability increased Michaelis constants for G6P and NADP a small increase of electrophoretic mobility and a biphasic pH-activity profile. This variant, associated with a chronic haemolysis, differs from all those previously described and has been termed G6PD Velletri.

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Table I. Metabolic findings

	Patient	Normal values (40 subjects) mean ± SD
Enzymes IUB/10^{10} red cells		
HK	110	91 ± 25
PFK	2,000	1,328 ± 233
ALD	790	493 ± 107
TPI	85,176	64,532 ± 9619
GAPD	6,500	6,544 ± 1122
PGM +2,3 DPG	6,900	6,625 ± 1029
PGM -2,3 DPG	2,500	2,611 ± 754
ENOL	2,800	2,719 ± 624
PK	3,100	2,795 ± 289
LD	44,694	31,039 ± 4611
G6PD	50	1,074 ± 289
6PGD	1,700	1,081 ± 283
NADP-GR	510	301 ± 114
NADPH-GR	1,500	957 ± 122
Substances, mg/ml red cells		
GSH (before incubation APH)	0.40	0.74 ± 0.10
GSH (after incubation APH)	0.10	0.70 ± 0.10
NANA	0.0063	0.0016 ± 0.0003
AMP	0.026	0.02 ± 0.0059
ADP	0.095	0.05 ± 0.0068
ATP	0.53	0.57 ± 0.09
Heinz bodies, /m red cells	2,870	2,262 ± 132
Autohaemolysis, %	5.30	0.4/1.53
After incubation in glucose	1.84	
After incubation in ATP	3.86	

HK = Hexokinase PFK = phosphofructokinase ALD = aldolase TPI = triosephosphate isomerase GAPD = glyceraldehydtriphosphat dehydrogenase PGM = phosphoglyceromutase 2,3 DPG = 2,3 diphosphoglycerate ENOL = enolase PK = pyruvate kinase LD = lactate dehydrogenase 6PGD = 6-phosphogluconate dehydrogenase NADP-GR = NADP-glutathione reductase NADPH-GR = NADPH-glutathione reductase GSH = reduced glutathione APH = acetylphenylhydrazine NANA = N-acetyl-neuraminic acid.

Case Report

The propositus is a Caucasian male, born in Velletri, Italy on December 8, 1929 Grandparents and father were negative for haemolytic diseases. Mother died aged 76 by a cardiopathy and had suffered throughout her life from favism. Brothers and sisters all negative One of his daughters suffered from an episode of jaundice, at the age of 8, lasting for about 12 days.

When he was 42 years old, he had a cholecystectomy for gallbladder stone. The following year blood tests, taken before an operation for abdominal hernia, revealed an increase in serum bilirubin and reticulocytes. He was transferred to the Haematology Department for investigation of the persistent haemolysis. On physical examination, besides jaundice of the sclera, mild hepatosplenomegaly was present.

Haemoglobin 11.8 g%, haematocrit 36%, red cell count 3.7×10⁶/μl, and reticulocytosis of 7%. Leucocyte count 5 600/μl with 67% neutrophils, 5% eosinophils, 2% monocytes, and 26% lymphocytes. Platelet count 180×10³/μl The bone marrow showed an accelerated erythropoiesis without pathological cells. The osmotic fragility of fresh blood was within normal limits. The Coombs test was negative, and cold agglutinins were not present. No abnormal haemoglobin could be demonstrated on electrophoresis and haemoglobin stability was normal. Hb F 1.7%, and Hb A_2 3.55% of the total haemoglobin. Total serum protein 8.5 g/100 ml with normal electrophoretic pattern. The erythrocyte ^{51}Cr half-life was 9.5 days (normal range 27–30 days) with an increase in daily haemolysis to 3.2% (normal value 0.83%) Blood volume 5 700 ml (theoretical value 4 800 ml) volume of the erythrocytic mass 2,200 ml (theoretical value 2,200 ml), plasma volume 3,500 ml (theoretical value 2,600 ml). The indirect calculation of erythrocyte production in bone marrow revealed a condition of equilibrium between haemolysis and production. Normal values in spleen, liver and sacral area.

Erythrocyte Biochemistry

As seen in table I, red cell G6PD activity was reduced to 50 U/10¹⁰ red cells. Also, we demonstrated a constant and significant decrease of GSH and a considerable increase of Heinz bodies after incubation with acetylphenylhydrazine. The autohaemolysis test showed increased haemolysis (5.3%) at 48 h, which was reduced to 1.84 % by addition of glucose. With regard to other erythrocytic enzymes and metabolites [2, 3], higher values than normal were found for phosphohexokinase, aldolase, lactic dehydrogenase and NADPH glutathione reductase. On the contrary normal values were recorded for triosephosphate isomerase, glyceraldehydephosphate dehydrogenase, phosphate glyceromutase, enolase, 6-phosphogluconate dehydrogenase, acid phosphatase and for N-acetyl-neuramic acid, ATP ADP and AMP

Kinetic Studies of G6PD

As shown in table II, the G6PD of our case presents the following features, an electrophoretic mobility similar to, or slightly higher than the normal enzyme: a decreased affinity for G6P and NADP demonstrated by high Michaelis constants

Table I Metabolic findings

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Table II Metabolic studies of G6PD

	Patient	Gd (+) B
Activity µM%/ml red cells/min	30	140±18
Electrophoresis, % of normal	100–105	100
K_m G6P µM	140	38–55
K_m NADP µM	4 4	1.5–3.5
K_i NADPH, µM	30	40–60
Utilization of substrate analogues, % of normal substrate		
2d–G6P	33	6
Gal-6P	30	6
Deamino-NADP	330	40–60
pH activity profile	biphasic (6–10)	truncate
Thermostability	very unstable	thermostable

Gd (+) B = Normal variant of G6PD K_m G6P = Michaelis constant for glucose-6-phosphate K_m NADP = Michaelis constant for NADP K_i NADPH = NADPH dissociation constant NADP = nicotinamide adenine dinucleotidephosphate (oxydized) NADPH = nicotinamide adenine dinucleotidephosphate (reduced) 2d–G6P = 2-deoxy-glucose-6-phosphate Gal-6P = galactose-6-phosphate.

a decreased NADP dissociation constant; an abnormal utilization of the substrate analogues 2d–G6P Gal-6P and deamino-NADP· an approximately biphasic pH activity curve with two maximum peaks at pH 6 and pH 10, and finally a thermoinstability of the molecule.

Since the patient's discharge, repeated examinations showed a mild anaemia and reticulocytosis with normal bilirubin and iron in the serum. The value of G6PD shows no variation, neither are there acute episodes of anaemia, jaundice or haemoglobinuria. In two successive checks, the red cell ^{51}Cr half-life was 19 days.

Discussion

The variant of G6PD described in the present paper has a number of properties which appear to be unique and which distinguish it from other variants of the group producing congenital non-spherocytic haemolytic anaemia [5 6] Our patient tolerated chronic haemolytic anaemia well and during the years of observation we never observed any episodes of acute loss of red cells nor of jaundice.

The G6PD variants with decreased affinity for the substrate (G6P

and NADP) and increased inhibition by NADPH have a markedly unfavourable effect on the intro-erythrocytic conditions characterized by the low concentration of NADP and the high concentration of NADPH [6].

The presence of the variants of red cell G6PD with unfavourable kinetic properties, can explain the discrepancy that sometimes exists between clear anaemic manifestations and the small enzymatic deficit. While the haemolytic symptomatology of some of the variants of G6PD like Gd-Bat Yam or Gd-Ramat-Gan and Gd-Worcester can be easily correlated with the lack of activity, this correlation is missing in other cases. For example, the Gd-Union, Gd-Markham, and Gd-Mediterranean variant are associated to a severe enzyme deficiency but have not been reported to be associated with any haemolytic symptomatology. On the contrary, other variants, such as Gd-Manchester, Tripler and Alhambra, with a moderate or mild enzyme deficiency, have been reported to be associated with a severe haemolytic anaemia. The G6PD of our patient can be considered as a variant with unfavourable kinetic properties because of the low affinity for the substrate G6P and for the co-enzyme NADP, and of the increased inhibition by NADPH (i.e., decreased K_i NADPH). The severe enzyme deficiency (<10% of normal) and unfavourable kinetic charges seem to be correlated with the chronic haemolysis of the patient.

However, in our patient it is of particular interest to point out the normal values in red cells of adenine compounds, and specially of ATP, and the increased activity of red cell NADP-glutathione reductase, which could be the expression of a mechanism of compensation for the decrease of G6PD, and consequence for the decrease of NADPH. The variant described here thus appears to differ from those previously described, and it is proposed to call it G6PD Velletri.

Acknowledgements. The kinetic studies of G6PD were carried out at the Centre de Recherches sur les Enzymopathies Hôpital Beaujon, Clichy.

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The G6PD variants with decreased affinity for the substrate (G6P

Book Reviews Buchbesprechungen Livres nouveaux

O. N. Ulutin (ed.): Platelets. Recent Advances in Basic Research and Clinical Aspects. Excerpta Medica, Amsterdam 1975 X + 526 pp., Dfl 170.–/ US $ 70.95. ISBN 90-219-0290-7

Under the title *Platelets*, Ulutin edits the papers presented at an international symposium on blood platelets held in Istanbul, from August 24 to 27 1974. The numerous papers are organized into five different groups. The first and most important part of these proceedings concerns the physiology biochemistry and ultrastructure of platelets as well as their adhesion, aggregation and secretory functions. Despite the unavoidable fact that Congress communications are of greatly variable quality and originality the first section has the great advantage to include experimental data and methods, which are absent in review articles, and extensive lists of references.

The second section is reserved to the platelet diseases and limited to few descriptive papers and to some on diagnostic methods.

The third part brings together various aspects of the participation of platelets in the pathologic processes of thrombosis and disseminated intravascular coagulation, their behaviour in atherosclerotic patients and long after myocardial infarction or deep vein thrombosis. The medicamentous alteration of the platelet functions is essentially limited to *in vitro* experiments.

In the fourth part, four papers deal with von Willebrand's disease.

The last part brings together papers on various subjects, including few on idiopathic thrombocytopenic purpura.

These proceedings are excellently presented. However subject index would have facilitated the search for special informations and references. Ulutin may be congratulated for the organisation of the Istanbul Symposium and for the work he has accomplished editing its proceedings. F. Duckert *Basel*

D. E. G. Austen and I. L. Rhymes (eds.): A Laboratory Manual of Blood Coagulation. Blackwell, Oxford 1975 XI + 110 pp.; £ 3.50. ISBN 0-632-00781-8.

Austen and Rhymes publish laboratory manual of blood coagulation which describes the methods and techniques used at the Oxford Haemophilia Center. It is al ays quite interesting to possess collection of all methods in force in a particular center. The manual is easy to consult, the descriptions are clear and easy to follow. One misses, however the references to original articles, which certainly give more details on the methods themselves, for example on reproducibility sensibility. The chapters on the calculation of activities are more complicated and somewhat difficult to understand. Even though the methods cannot be adopted in every laboratory the manual is useful guide for all scientists interested in coagulation studies.

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S. N WICKRAMASINGHE. Human Bone Marrow Blackwell, Oxford 1975. XIV+458 pp., £ 16.50.

Der Titel des Buches mag täuschen, es handelt sich nicht um eines der klassischen, vorwiegend der Morphologie gewidmeten Werke. Im Zentrum steht vielmehr das funktionelle Geschehen. Folgerichtig beginnt der Autor *ab ovo* mit einer Darstellung der Eiweisssynthese sowie der Methoden zur Erfassung der Zellproliferation im allgemeinen, um sodann die fetale Blutbildung und die anschliessenden Veränderungen zu besprechen. Das folgende Kapitel handelt von der Organisation des Knochenmarkes, womit wiederum der funktionelle über den rein morphologischen Aspekt gestellt ist. Ausführliche Kapitel sind den hämopoetischen Stammzellen, der neutrophilen Granulopoese, der Erythropoese, der Produktion der übrigen Leukozyten und Makrophagen sowie der Megakaryopoese gewidmet, wobei jeweils der Hauptakzent auf der Zellkinetik und den Regulationsmechanismen liegt. Nachdem sich nahezu 300 Seiten mit der normalen Physiologie befassen, werden auf den verbleibenden 90 Seiten einige pathologische Zustände der Zellproliferation herausgegriffen und näher beleuchtet. Besondere Beachtung verdienen die Ausführungen zur megaloblastären Hämopoese, hat doch der Autor auf diesem Gebiet selbst wichtige Beiträge geliefert. Daneben gibt er einen umfassenden Überblick über die Literatur bis etwa 1973 (64 Seiten Literaturverzeichnis).

Gesamthaft enthält das Buch eine Fülle von Informationen über ein höchst aktuelles Gebiet der Hämatologie. Es bietet nicht nur dem experimentell aktiven Biologen, sondern auch dem klinisch tätigen, an den Grundlagen des Faches interessierten Arzt sehr viel Wissenswertes in einer übersichtlichen, gut verständlichen Form.

U BUCHER, *Bern*

S. M LEWIS and J F COSTER Quality Control in Haematology Symposium of the International Committee for Standardization in Haematology Academic Press, London 1975 XII+240 pp., £ 7.50/US$ 19.50.

Im vorliegenden Band äussert sich eine Reihe hervorragender Experten zum Thema Qualitätskontrolle. Grossenteils handelt es sich um Arbeiten, welche anlässlich eines Symposiums im Rahmen des Internationalen Hämatologenkongresses im *September 1974 in Jerusalem vorgetragen worden sind. Nach einer vorzüglichen* Einführung, in welcher R. EILERS sechs Phasen der Qualitätskontrolle umschreibt, referieren verschiedene Autoren über Inter Laboratorien-Versuche auf internationaler und nationaler Ebene Sodann wird das Problem der Standards von verschiedenen Seiten beleuchtet (u. a. auch Standardisierung der Farbstoffe sowie der Antikoagulantien-Überwachung). Weitere Beiträge befassen sich mit dem Erythrozytenvolumen und dem Hämatokrit, ferner mit der laborinternen Qualitätskontrolle, der Kontrolle qualitativer Tests usw Die Praxisnähe wird durch die beiden letzten Kapitel (Probengewinnung und Kosten der Qualitätskontrolle) unterstrichen. Es ist sehr zu hoffen, dass das Buch Eingang in möglichst viele klinische Laboratorien findet. Qualitätskontrolle kann heute nicht mehr nur als Hobby einiger weniger Laboratorien aufgefasst werden. Gerade der vorliegende Band vermag die breiten Anwendungsmöglichkeiten klar aufzuzeigen. Auch der Routinierte wird mit Gewinn darin lesen.

U BUCHER, *Bern*

Acta haemat. 57: 129–134 (1977)

Freeze-Fracture of Circulating Human Eosinophils[1]

STEVEN D. DOUGLAS and MARY P. OOKA

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Key Words. Electron microscopy · Eosinophils · Freeze-fracture · Granules · Plasma membrane

Abstract. Freeze-fracture has been carried out on peripheral blood from three patients with eosinophilia. Eosinophil granule membranes are characterized by intramembranous particles (IMP) with diameters of 80–150 Å. These particles of the granule membrane are consistently larger than the IMP of the plasma membrane which are approximately 80 Å in diameter. Cross-fractures of granules showed no evident subgranular inclusions. The difference in size between granule and plasma membrane particles observed in freeze-fracture suggests that studies of eosinophils may be an approach to membrane alterations during phagocytosis.

The mature eosinophil derived from the myeloid series of the bone marrow is characterized by a unique granule type which contains a crystalloid and several lysosomal enzymes [1, 7–10]. Recent evidence has also suggested the existence of a second small granule type in the eosinophil [4]. This cell type is capable of active pinocytosis and phagocytosis. Cell separation techniques have not made possible the isolation of purified population of eosinophils, however, the occurrence of individuals with eosinophilia has led to its structural and functional characterization [9, 10]. By scanning electron microscopy it is difficult to distinguish eosinophils from other leukocytes [6]. Since eosinophils are characterized by a unique crystalloid-containing granule, the freeze fracture technique is an approach to further morphologic study of the structure of the mem-

[1] This work was supported by the National Institutes of Health, USPHS grant No. AI 1 478 and the National Leukemia Association, Inc.

branes of this granule type. Accordingly we have investigated the freeze-fracture morphology of the circulating human eosinophil

Materials and Methods

Heparinized (10–20 U Upjohn/ml) peripheral blood was obtained from three patients with marked nonleukemic eosinophilia [10] Buffy coats were prepared by centrifugation of whole blood at 1,000 rpm for 8 min. The cells were resuspended in Hanks balanced salt solution and differential counts were performed on Wright Giemsa smears. The cells were pelleted and a portion of the pellet was fixed with cacodylate-buffered 1.5% glutaraldehyde, postfixed in 1.0% buffered osmium tetroxide, dehydrated and embedded in epon and sectioned with LKB ultrotome III.

For freeze-fracture studies cells were fixed in 1.5% glutaraldehyde in 0.1 M PBS, pH 7.4 at 27 °C for 15 min. The cells were then washed three times in the same buffer and resuspended in 1 ml of the buffer 25% glycerol in 0.1 M PBS, pH 7.4 was added dropwise at 37 °C until a total volume of 15 ml was reached The tubes were centrifuged, the supernatant discarded, and the cells resuspended in fresh 25% glycerol at 37 °C. They were kept at this temperature for 30 min and then placed at room temperature for 30 min. The cells were frozen on gold stubs in Freon 22, cooled over liquid nitrogen and introduced into Balzers 301 Freeze Etch Microtome at –150 °C.

The cell pellets were fractured at –100 °C and shadowed with platinum-carbon followed by carbon [5] The replicas were cleared with sodium hypochlorite. The specimens were examined with a Siemens 102 electron microscope operated at 80 kV

Results

More than 70% of the cells examined for each preparation in thin section were typical eosinophils which contained mature crystalloid contained granules. Freeze fracture of the plasma membrane of eosinophils revealed typical A and B faces with the characteristic 80 Å intramembranous particles (IMP) (fig 1–4) Fractures through nuclei revealed characteristic IMP and nuclear pores Aggregation was not observed for either plasma membrane or nuclear membrane IMP The characteristic A (external aspect of the inner face) and B (internal aspect of the outer leaflet) faces of the plasma membrane could be identified. Fracture of the eosinophil granules also revealed characteristic faces. In accord with the nomenclature proposed previously for eosinophils, mast cells and granules in other cell systems [2, 3] the fracture face of the outer cytoplasmic leaflet of the granule is designated as the A face and that of the inner leaf

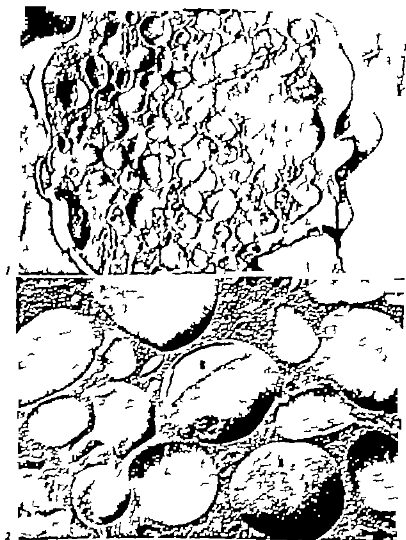


Fig 1 Platinum-carbon replica of eosinophil. The B face of the plasma membrane is evident. There are numerous cytoplasmic granules which reveal A and B faces. 1[illegible],000

Fig 2 Higher magnification of platinum-carbon replica showing the characteristic faces of the eosinophil granule membrane (A and B). There is some aggregation of IMP on the A face. 45,000.

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let as the B face. The A face of the granule contains a greater number of IMP than the B face (fig. 2). The IMP associated with the granule membrane measure 80–150 A in diameter. In some fractures aggregation of granule membrane IMP was observed on the A face (fig. 2). Cross-fractures of granules failed to show any characteristic structures (crystalloids could not be identified). The second granule type [4] could not be identified in freeze-fracture replicas.

Discussion

Freeze-fracture studies of circulating human eosinophils from three individuals with eosinophilia demonstrated the characteristic structure of the plasma membrane and nuclear membrane systems. The topography of the faces of the eosinophil granules is characterized by larger (80–150 A). IMP than the IMP associated with the plasma membrane. Occasional aggregates of IMP were observed in the A face of the granule. CHI *et al.* [3] in a study of the rat peritoneal mast cell have reported cylindrical protuberances and invaginations in the plasma membrane; these have not been observed for the eosinophil. The subgranular inclusions reported by others in mast cells and eosinophils using freeze-fracture [2] were not observed in this study. The eosinophil has not as yet been studied by freeze-fracture during active pinocytosis or phagocytic activity. The present study of the resting eosinophils suggests that the investigation of this cell type during phagocytosis may afford a possible approach toward characterization of the topologic events which occur during engulfment and during granule-phagocytic vacuole fusion. The observed size differences between plasma membrane and granule IMP is of interest. Its functional and dynamic significance requires further investigation.

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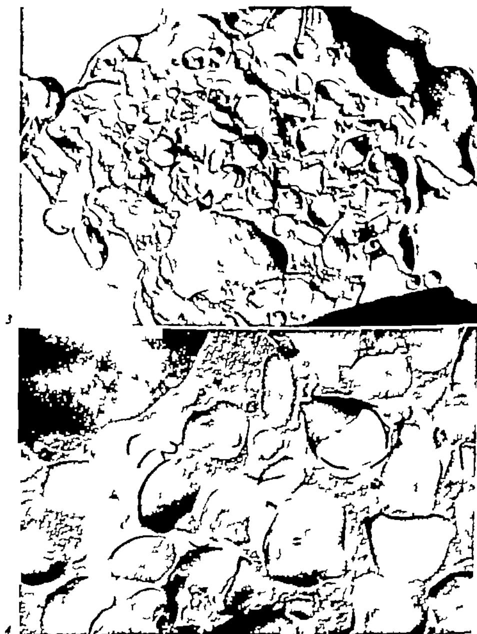


Fig 3 Freeze-fracture platinum-carbon replica of another eosinophil × 12,000

Fig 4 Higher magnification of cell shown in figure 3 Note that the particles associated with granules are larger than those on the plasma membrane × 45,000.

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Circulating Anticoagulant against Factor XII and Platelet Antibodies in Systemic Lupus Erythematosus

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Key Words. Anticoagulants · Anti-platelet antibodies · Bleeding disorders · Factor XII · Lupus erythematosus · Platelet aggregation

Abstract. A case of systemic lupus erythematosus is reported, with circulating anticoagulant against factor XII, associated with impaired platelet aggregation, presumably in relation to the presence of anti-plat let antibodies.

Patients with systemic lupus erythematosus (SLE) frequently suffer from severe blood coagulation disorders. The bleeding symptoms are due to thrombocytopenia, circulating anticoagulant, vascular fragility or to clotting factor deficiency [4 26].

We have studied a patient with SLE. After a long recurrent thrombocytopenia, she had slight bleeding symptoms from an anticoagulant directed against factor XII. Cronberg and Nilsson [11] also described this inhibitor in a patient with SLE but our case is different because this anticoagulant was associated with impaired platelet aggregation.

Methods

Coagulation studies. Bleeding time [18], coagulation time in glass tubes and in silicone-treated tubes, recalcification time, partial thromboplastin time (PTT), prothrombin time, thrombin time, clot retraction, thromboelastogram (TEG), euglobulin clot lysis time, fibrinogen and one-st ge assay for factors II XII were d termined (for methods, see table I). Factor XIII was estimated by the qualitative test of clot solubility in [illegible] monochloracetic acid and the fibrin degradation products (FDP) by the method of Merskey *et al.* [4].

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Platelet investigations. The platelets were counted by the method of BRECHER and CRONKITE [6]. Spontaneous platelet aggregation was studied by the method of BREDDIN [7]. Platelet aggregation induced by collagen (Sigma: 40 μg/ml), ADP (Boehringer: 1 μM/ml), adrenaline (Merck: 1 mM/ml), Thrombofax (Ortho) and spontaneous aggregation after incubation of platelet rich plasma (PRP) at 4, 4 37 C for 30 min were estimated by the method of BORN and CROSS [5] with the platelet aggregometer EEL 169.

After aggregation, platelet factor 4 release was determined by procedure of HARADA and ZUCKER [14]. Platelet factor 3 (PF3) availability was estimated after PRP incubation with kaolin according to WEISS [28].

Anti-platelet antibody was determined by three techniques: the method of PF3 availability [19], the optical density changes in aggregometer after incubation of normal PRP with test serum [12] and the platelet serotonin release test [16].

Inhibitor investigations. Inhibitory activity present in the patient's plasma was determined by PTT: 1 part of normal plasma was incubated with 1 part of the patient's plasma for 60 min at 37 C. This method was used also for the determination of factors XI and XII. The possible inhibitory activity against intrinsic thromboplastin activity was determined by thromboplastin generation test (TGT) and against extrinsic thromboplastin activity by the incubation of test serum with increasing dilutions of tissue thromboplastin. Inhibitor against the contact activation product was determined by a modification of the method of NOSSEL [25].

Case Report

The patient was a 4-year-old woman. In 1956, erythema appeared progressively on her face, periorbital regions, nose, hands and feet. A clinical diagnosis of discoid lupus was made and the patient was treated with chloroquine. In 1963, in association with menarche, profuse uterine bleeding occurred. The platelets were 65 000/μl; anti platelet antibody, LE cells, rheumatoid factor could not be observed. In 1964 tonsillectomy was performed because of chronic tonsillitis and pleuritis on the left side. In 1968, phlebitis occurred in the left internal saphena.

During the following years, the patient had recurrent arthralgia and persistent erythema. Laboratory findings revealed recurrent thrombocytopenia, negative LE test, rise of lymphocytes, histiocytes and plasma cells in the bone marrow and increased γ-globulin level. During this period, the patient was treated with chloroquine, acetyl-salicylic acid and corticosteroids.

The last physical examination revealed erythema with confluent scaly mottles on the fingers and on forehand, with hypotrophia of the subcutis.

The results of *laboratory investigations* are reported in table I.

Serum immunoelectrophoresis: increase in IgG. Serological investigations revealed no increase in the titer of antistreptolysin or rheumatoid factors. Antinuclear factors were present in a high titer (1/64). LE cell preparations were negative. However, according to the criteria of COHEN and CANUSO [10], we think the patient was affected by SLE because five clinical signs of this disease were present (thrombocytopenia, pleuritis, arthritis, discoid lupus, facial erythema) and antinuclear antibodies research was positive, in spite of negative LE cell preparation.

Table I. Laboratory investigations

ESR 1K	55	Urinalysis	ne	Albumin %	53.7
RBC/µl	3,800,000	Blood urea nitrogen g%	0 70	-Globulin %	3.8
Hb, g%	12.3	Blood glucose mg	0 50	α_2-Globulin, %	7.5
WBC/µl	5,000	Serum bilirubin, mg	0 45	β-Globulin %	10 4
N, %	46	Direct, mg%	0 30	γ-Globulin, %	4 2
E, %	2	Indirect, mg%	0 15	Total serum protein, g%	7.2
M, %	4				
L, %	48				

Table II. Coagulation studies

Test	References for methods	Patient's plasma	Normal values
Bleeding time, min	18	14	6–12
Coagulation time	8		
In glass tubes, min		18	6–14
In silicone-treated tubes, min		25	12 25
Recalcification time sec	8	160	80–120
PTT sec	22	80	30–45
Quick time, sec	8	13	1 14
Thrombin time sec	8	15	13 17
Clot retraction, %	23	54	45–64
TEG	15		
r, mm		12	6–10
k, mm		11	7 10
ma, mm		50	50–68
Fibrinogen mg/100 ml	9	780	200–400
Factor II, %	8	95	80–120
Factor V %	8	70	80–170
Factor VII,	8	100	80-120
Factor VIII %	8	85	60–160
Factor IX, %		60	60–160
Factor X, %	1	100	80–120
Factor XI	17	85	60-120
Factor XII	3	45	60 120
Factor XIII	11	normal	normal
Euglobulin lysis time min	20	120	120
FDP µg/ml	4	10	0–40

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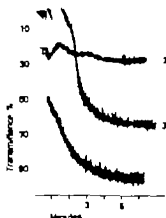


Fig 1 Modifications of platelet aggregation induced by the patient plasma. 1 = Normal PRP aggregation by ADP. 2 = aggregation wave by ADP after incubation of normal PRP with patient' platelet poor plasma 3 = normal PRP aggregation by Thrombofax, 4 = aggregation wave by Thrombofax after incubation of normal PRP with the patient's plasma.

Table IV Inhibitory activity Modifications of standard plasma PTT (sec) after incubation with patient plasma (37 °C for 30 min)

Standard plasma	37
Patient's plasma	80
Standard plasma (1 ml) + absorbed $BaSO_4$ pathological plasma (0 40 ml)	64
Standard plasma (1 ml) + heated pathological plasma (56 °C for 5 min 0.40 ml)	64
Standard plasma (1 ml) + test serum (ml 0.40)	62

Platelet investigations. Spontaneous platelet aggregation [7] was normal. When the PRP was tested on Born and Cross [5] aggregometer spontaneous aggregation could not be demonstrated. After incubation of patient's PRP at 4, 24 and 37 °C with collagen, ADP adrenaline and Thrombofax in the aggregometer changes in optical density were not observed. After aggregation we did not find any release of anti-heparin activity PF3 availability test was negative.

The research of anti-platelet antibodies by the method of PF3 availability was negative. Therefore, we have studied platelet aggregation of normal plasma before and after incubation with the patient' plasma, in this manner it was possible to demonstrat an inhibitory effect of test plasma on normal platelet aggregation (fig. 1). Using the serotonin release test, we obtained very high positivity In

Table III Study of the circulating anticoagulant

Thromboplastin generation test sec	
Normal plasma as substrate	47 29 13
Pathological plasma as substrate	65 39 25 11
Quick time with increasing dilutions of tissue thromboplastin sec	
Normal plasma	
Whole thromboplastin	16
Diluted 1/2	18
Diluted 1/5	2[illegible]
Diluted 1/10	28
Pathological plasma	
Whole thromboplastin	17
Diluted 1/2	19
Diluted 1/5	[illegible]3
Diluted 1/10	31
Contact activation test sec	
Intact normal plasma	122, 148 203
Intact pathological plasma	193, 282, 345
Control without contact product	497

Incubation mixtures for the research of an inhibitor directed against factor IX or factor XII	
Normal plasma	
Factor IX,	100
Factor XII	100
Pathological plasma	
Factor IX,	85
Factor XII	32
Mixture 1 1	
Factor IX, %	
After 0 min	85
After 60 min	90
Factor XII %	
After 0 min	3
After 60 min	32

Coagulation st d es. Platelets were of normal number coagulation time in glass tubes, recalcification time and PTT were slightly prolonged (table II). The plasmatic concentrations of factors XI and XII were low The findings of the other factors, FDP euglobulin lysis time, TEG clot retraction were normal.

The addition of the patient s plasma to normal plasma (1 part of patient s plasma was incubated with 1 part of normal plasma) prolonged the PTT (table III) The TGT with patient s plasma as substrate was negative and no anticoagulant against extrinsic thromboplastin activity could be demonstrated.

The celite eluate test for Hageman factor and factor XI deficiency [5] used with the patient plasma as 'non-contact plasma to demonstrate normal product contact plasma activity on the 'intact test plasma revealed the inhibitory effect of the patient s plasma on the control plasma activated by celite.

By the determination of single clotting factors, an anticoagulant activity has only been demonstrated against factor XII whose concentration was 30%. The inhibitor was not adsorbed to BaSO and was present in serum and its activity resisted 56 °C (table IV)

gations revealed a circulating anticoagulant in only 3 patients, who had low levels of factors II V VII and IX but none of them showed an inhibitor against factor XII

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fact, the control samples showed a supernatant radioactivity of 4,861±698 cpm. The released radioactivity of normal platelets after incubation with test serum was 28,000 cpm.

Discussion

The disease in our patient began when she was 8 years old. In the first years of the disease, she had erythema and thrombocytopenia then she had pleuritis, phlebitis in the left leg and the erythema increased In this period antinuclear factors were never present.

When we studied the patient, she had slight bleeding symptoms, arthralgia in the phalanges and in the metacarpus. The clinical severity of the disease was correlated with impaired platelet function The coagulation investigations showed the presence of an inhibitor directed against Hageman factor present in serum, not adsorbed to $BaSO_4$ thermostable We observed no inhibitory effect against intrinsic and extrinsic thromboplastin activity

Due to the presence of anti platelet antibody the platelet could not be aggregated by ADP collagen adrenaline, or Thrombofax. We cannot say if the inhibitory effect against Hageman factor and platelet aggregation is caused by the same protein or by two different antibodies

The circulating anticoagulants are a characteristic finding of SLE and at least 40 cases have been described These inhibitors which are immunoglobulins IgG or IgM were sometimes associated with thrombocytopenia and were frequently directed against prothrombin activation More rarely the anticoagulants were active to other blood coagulation factors, as factors VIII IX XI or Hageman factor Therefore, our case is characterized by inhibitor activity against factor XII and impaired platelet aggregation and thus represents an uncommon type of hemorrhagic disorder in SLE. In the literature there are similar cases but they showed different aspects. CRONBERG and NILSSON [11] described a woman with SLE and an anticoagulant directed against factors XI and XII but in contrast with our case the platelets showed an intense spontaneous aggregation

Recently REGAN *et al* [26] examined 50 patients with SLE. They found a qualitative defect absence of collagen induced aggregation and impaired ADP and adrenaline induced aggregation Our findings are in agreement with theirs. This defect seemed to result from an anti platelet factor detectable in the serum of some patients. The coagulation investi

gations revealed a circulating anticoagulant in only 3 patients, who had low levels of factors II V VII and IX but none of them showed an inhibitor against factor XII.

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Congenital Heinz Body Haemolytic Anaemia Due to Haemoglobin Perth in a Nama Child Seemingly Aggravated by the High Nitrate Content of the Water Supply

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Key Words. Haemoglobinopathies Hb Perth Haemolytic anaemia Heinz bodies Nitrat effect on haemoglobin Unstable haemoglobins

Abstract. A Nama child from South West Africa (Namibia) with very severe congenital haemolytic anaemia has an unstabl haemoglobin (Hb Perth). The severity of the condition seems to have been aggravated by the high nitrate content of the water supply

Congenital Heinz body haemolytic anaemia (CHBHA) is the generic term for a group of anaemias in which the haemoglobin precipitates within the red cell producing insoluble inclusions known as Heinz bodies. It was not until 1966 that a case of CHBHA was shown to result from a specific structural abnormality namely Hb Köln due to the substitution of methionine for valine at position 98 of the β-chain of globin [1] Subsequently the amino acid substitution has been defined in over 50 different unstable haemoglobins [2]. The overwhelming majority of cases of moderate to severe unstable haemoglobin disease are sporadic and are not present other family members among the most severe they are all sporadic.

In this communication we report a sporadic case of severe CHBHA occurring in Nama boy from Namibia (South-West Africa) due to an unstable haemoglobin previously characterised and called Hb Perth [3] and Hb Abraham Lincoln [4]. The severity of the anaemia was particularly striking and enquiry revealed that the nitrate content of water from

the wells used by the Nama people is high and might play a part in determining the severity of the haemolysis

Methods

Haematological values were determined by standard methods [5] and using the Coulter Model S. For haemoglobin studies, blood samples were collected into ACD solution and transported on wet ice to the laboratory. Cells were washed 3 times in isotonic saline and lysed with vol of cold water or carbon tetrachloride and the stroma removed by centrifugation. Haemoglobin electrophoresis was carried out in cellulose acetate using a Tris-EDTA-borate buffer pH 8.9 and in starch gel using the same buffer system [?] HbA_2 determinations were carried out by elution of the component separated on cellulose acetate and HbF estimation made as described by BETKE et al [6]. The heat stability test was carried out according to the methods of DACIE et al [7] and CARRELL and KAY [8]

The precipitate obtained by CARRELL and KAY's method was first dissolved in 0.1 N HCl containing 5 M urea, before removing the haem with HCl/acetone at 20 C, according to ROSSI-FANELLI et al [9] Chain separation was done according to CLEGG et al [10] The chains were aminoethylated according to JONES and HUISMAN [11] before recovery by extensive dialysis against 0.5% formic acid and freeze-drying. They were then digested with TPCK trypsin (Worthington Biochemicals Corp Freehold, N.J.), 2 and 4-mg portions of tryptic digests were applied to Whatman No 3 paper for diagnostic and preparative purposes, respectively for finger-printing in a method already described [12]. Diagnostic finger prints were stained with 0.2% ninhydrin (Merck, Darmstadt, FRG) in acetone and stained for methionine, histidine, arginine, tryptophan and tyrosine using reagents summarised by LEHMANN and HUNTSMAN [2] in the order outlined by EASLEY [13]. Preparative finger prints not stained with ninhydrin but with tryptophan were identified under ultraviolet light. The abnormal peptide was purified by electrophoresis at pH 3.5, then hydrolysed in 6 N HCl at 108 C for 48 h. Amino acid composition was determined using a Locarte amino acid analyser. Dansyl Edman degradation of the peptide was carried out as described by GRAY [14] the dansyl derivatives being identified on polyamide layers using the solvents of WOODS and WANG [15].

Case Report

A 13-year-old Nama (Hottentot) presented in May 1973 with a history of many years of weakness, pallor and the passing of d k urine. He had received many blood transfusions. 2 years previously his spleen had been removed but no improvement followed. From the history it seemed as though chest and upper respiratory tract infections triggered off haemolytic episodes. Neither parent of the proband has suffered from anaemia or jaundice and there is a 12 year-old brother who also gives no history of serious illness.

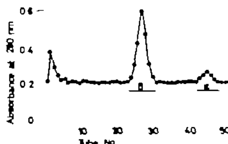


Fig. 1. Elution pattern of the precipitate obtained by incubating whole haemolysate with isopropanol at 37 °C after removal of haem on CM-23 cellulose acetate column (1.5×22 cm) at room temperature. Buffers were prepared in 8 M urea-2 mercaptoethanol (0.005–0.03 M $Na_2HPO_4 \cdot 2H_2O$) and the pH adjusted to 6.7 with H_3PO_4. 6-ml fractions were collected at a flow rate of 60 ml/h.

Haematological investigations. A blood specimen obtained from the child, prior to transfusion, gave the following values: Hb 5.1 g/100 ml, haematocrit 17%, erythrocyte count 1.6 $10^6/\mu l$, MCV 103 μm^3, MCH 32 pg, MCHC 32%, nucleated erythrocytes 40,000/μl, reticulocytes 50%. The morphology of the red cells was typical of severe haemolytic anaemia due to an unstable haemoglobin in an individual who has undergone splenectomy: anisopoikilocytosis, marked fragmentation, punctate basophilia, target cells, Howell-Jolly and Pappenheimer inclusions. Supravital staining produced Heinz bodies in nearly all the cells. The cells treated by the technique of KLEIHAUER *et al.* [19] were of very striking appearance because many contained large globules of red-coloured material. These, presumably represented masses of precipitated haemoglobin. In the supravitally stained preparations, it was not easy to distinguish between reticulocytes and the other cells with inclusion bodies but it appeared as though about 50% of the cells were, in fact, reticulocytes. The red cell glucose-6-phosphate dehydrogenase (G6PD) level was elevated.

Denaturation and precipitation of the haemoglobin was produced when a 1/25 dilution of haemolysate was heated in iso-osmotic phosphate buffer pH 7.4, for only 15 min at 50 °C. This instability was also demonstrated when the haemolysate was heated in 17% isopropanol in Tris-HCl buffer.

Haemoglobin Studies

No abnormal haemoglobin components could be demonstrated by cellulose acetate or starch-gel electrophoresis but the proportion of HbA_2 was raised (6.3%) as was the HbF (11.7%) (table I). CMC chromatography of the precipitate prepared by CARRELL and KAY's method [8] showed that the β-chain had been selectively precipitated (fig. 1).

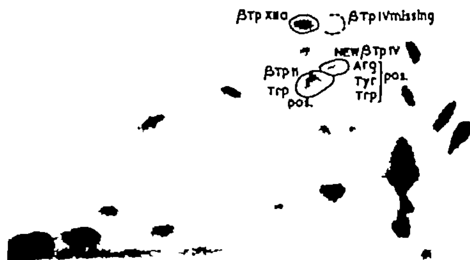


Fig. 2. Finger-print pattern of the soluble tryptic peptides of the aminoethylated β-chain of Hb Perth.

The finger prints of the aminoethylated β-chain showed on staining with ninhydrin that peptide β^ATpIV (residues 31–40) which has the same chromatographic mobility as βXIIa (residues 105–112) was missing. A new peptide was seen running very close to βTpII (residues 9–17). The new peptide presumably the abnormal βTpIV stained for tryptophan tyrosine and arginine indicating that none of these residues was involved in the substitution (fig. 2). Since peptides βTpII and TpIV are both tryptophan-positive, they were detected under a long wave ultraviolet light on ninhydrin unstained preparative finger prints. They were purified by high-voltage electrophoresis at pH 3.5 then hydrolysed in 6 N HCl for 48 h rather than 24 h as βTpIV peptide contains a Val Val bond which requires longer time for hydrolysis to go to completion. The amino acid analysis of the new peptide is shown in table II. A residue of leucine is missing and instead of it there is found an extra residue of proline. Normal βTpIV (β31–32) has the sequence

Arg –	Leu –	Leu –	Val –	Val –	Tyr –	Pro –	Trp –	Thr –	Gln	Arg
30	31	32	33	34	35	36	37	38	39	40

and the replacement is therefore either at positions 31 or 32. The residue preceding β31 is one of arginine. It has been demonstrated that an arginine-proline bond is resistant to tryptic hydrolysis [16]. If the replacement

Table I Haemoglobin values in family of H. P (Hb Abraham Lincoln/Perth)

	Hb, g %	Heat stability	HbF %	HbA_2 %	G6PD
Proband	5.1	abnormal	11 7	6.3	↑
Father	14.5			–	–
Mother	13 4	normal	1.0	3.3	normal
Brother	13.0	normal	0.7	3.7	normal

Table II. Amino acid composition of βTpIV of proband

Amino acid	Nanomoles	Molar ratio	Expected for βATpIV
Threonine	35.2	0.87	1
Glutamine	39.2	0 97	1
Proline	89.8	2 20	1
Valine	78.2	1 92	2
Leucine	46.8	1 15	2
Tyrosine	34 9	0.86	1
Arginine	40 8	1.00	1
Tryptophan	+		1

was at β31 then a double peptide βTpIII IV would be formed. But such a peptide was not observed. Dansylation of the abnormal peptide demonstrated that leucine was the N-terminal residue and that the replacement of leucine was therefore at position β32.

Discussion

Proline residues are located in haemoglobin either in non-helical regions or within the first three positions of a helix. Introduction of proline into the middle of the helix, as in this case, causes its disruption because the shape of the heterocyclic ring of proline disturbs the helix, and causes instability of the haemoglobin molecule [17 18] Such substitutions by proline therefore cause haemolytic disease and this has been observed in Hb Bibba (136 Leu→Pro) [19] Genova (β29 Leu→Pro) [20] Santa Ana (β88 Leu→Pro) [21] Sabine (β91 Leu→Pro) [22] Casper/Southam-



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ton (β106 Leu→Pro) [23] Duarte (β62 Ala→Pro) [24] Perth/Abraham Lincoln (β32 Leu→Pro) [3 4] They are all unstable haemoglobins associated with varying degrees of haemolytic disease In the present patient, replacement of leucine at β32 by proline disrupts the B-helix thus breaking the contact between the adjacent leucine (β31) and the haem and probably breaking also the $\alpha_1\beta_1$-contacts at positions β30 33 34 and 35 in the same area of the β-chain Consequently an unstable haemoglobin haemolytic anaemia results as has been reported in Hb Perth/Abraham Lincoln [3 4] which has the same substitution at β32

The degree of anaemia suffered by our patient seems to be greater than in either of the 2 previously described patients with this same haemoglobin variant The case of JACKSON *et al* [3] had haemoglobin levels varying between 7 and 14 g/100 ml while the case of HONIG *et al* [4] had levels between 4 8 and 11 0 g/100 ml We sought therefore, some possible environmental factor which might aggravate our patient s clinical condition He lives on a farm many miles from main water supply and when we tested the nitrate content of the water used by the residents of the farm it was found that they were of an unusually high concentration 20 8 ppm Intestinal bacteria are capable of reducing nitrates to nitrites which on absorption readily oxidise haemoglobin to methaemoglobin With an unstable haemoglobin a raised nitrite concentration may aggravate the rate of methaemoglobin formation far in excess of the reductive capacity of the cells. This might be the case with our patient

The slightly elevated MCV in our patient is probably significant in view of the findings in the 2 previously reported individuals with this particular unstable haemoglobin variant [3 4] The MCHC is at the lower limit of normal and this is probably due to loss of intracellular haemoglobin as Heinz bodies [25]

Data obtained on the parents of the proband as well as one sibling show that this example of Hb Perth/Hb Abraham Lincoln is sporadic and has arisen by mutation in one of the gametes giving rise to him. With this severe degree of haemolysis, this is not at all surprising because an individual with this particular unstable haemoglobin would be highly unlikely to survive and reproduce. The patient from Perth Western Australia died in his early twenties the patient from Chicago is alive at 26 years of age but needs numerous blood transfusions.

Levels of HbF in the unstable haemoglobin syndromes are usually normal [26] although SCHNEIDER *et al* [22] found in their 18 year-old patient with Hb Sabine a level of 12% and WHITE and DACIE [26] report

ed that their 20-year-old patient with Hb Köln had a HbF level of 6%. JACKSON *et al* [3] do not report on HbF level in their patient with Hb Perth while HONIG *et al* [4] found a barely raised level (2.1 /) in their 26-year-old patient with the same variant. The level of 11 7%/ in our patient is, therefore, noteworthy and could, perhaps, reflect the more severe anaemia suffered by him in his 13 years of life due to the aggravating en vironmental insults referred to above.

Slight increases in the level of HbA_2 (up to 5) are, according to WHITE and DACIE [26] commonly found in heterozygotes for some of the unstable haemoglobin syndromes due to β-chain variants. They cite as ex amples of this finding the Hb Hammersmith [27] Philly [28] Sabine [22] and Shepherds Bush [29] but point out that this is not invariable, as demonstrated by Hb Bristol [25] HONIG *et al.* [4] found a HbA_2 lev el of 5.8%/ in their patient with Hb Abraham Lincoln and the patient presented here has an even higher level, 6.3 /%. The reason for elevated HbA_2 levels in patients with β-chain-unstable haemoglobins is not clear but it could be explained by loss of the unstable haemoglobin from the cells, as in the case of Hb Zürich.

An increased level of G6PD was found in the patient of HONIG *et al.* [4] and a similar situation exists in our patient. This is consistent with a young cell population but may also be due to increased requirements for reduced glutathione. They found levels of reduced glutathione of about 50% normal in their patient.

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Infectious Mononucleosis in Hodgkin's Disease

A Further Case Report

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Key Words. Epstein-Barr virus Hodgkin's disease Infectious mononucleosis

Abstract A patient who developed infectious mononucleosis while being treated for Hodgkin's disease is presented. This pathological profile does not support the concept of a causal relationship between Epstein-Barr virus and Hodgkin's disease.

The aetiology of Hodgkin's disease (HD) is still uncertain although several epidemiological studies have suggested an association between HD and preceeding infectious mononucleosis (IM) [3 11 13 14] While it is now widely accepted that the causal agent in IM is Epstein Barr virus (EBV) [5 15] the role of this virus and IM in relation to HD remains controversial Although initial studies [2, 9] showed significantly higher EBV antibody levels in patients with HD compared to controls, LANGENHUYSEN *et al* [8] using more sensitive laboratory techniques, subsequently demonstrated elevated antibody titres to both EBV and cytomegalovirus Serious doubts have therefore been cast on any specific or causal role for EBV in HD This view gains support from the few descriptions of the simultaneous presentation of the two disorders [4 10] and even more, from the recent and previously unreported development of IM in a patient who 3 years previously had undergone curative treatment for HD [7] The purpose of this brief communication is to report the occurrence of IM in a further patient with established HD

We acknowledge Dr J F BANATVALA, St Thomas Hospit l, London for measurement of the Epstein-Barr virus antibody levels.

Case Report

In June 1966, 24-year-old single girl presented with painless cervical lymph node enlargement involving the left posterior triangle. She was otherwise well and physical examination revealed no other abnormalities. X-ray of the chest and thoracic inlet were normal. While the peripheral blood findings were within normal limits, the ESR was slightly elevated at 20 mm/h. The ffected nodes were excised and their histology indicated chronic granulomatous reaction which was regarded as tuberculous in nature, although an atypical HD was not excluded. Repeated microscopy and culture of sputum and urine failed to demonstrate tuberculosis bacilli, but the patient was started on trial of nti-tuberculous therapy For few months she remained generally well, but subsequently developed an unproductive cough, and X-ray then revealed opacities in the right hilar and left mid zones, with sympathetic effusion at the left base. At this stage, the diagnosis of tuberculosis was re garded as no longer tenable, and in view of the previous histology and the emerging clinical picture, the possibility of typical HD was reconsidered Excision of enlarged lymph nodes from the right anterior cervical chain was therefore undertaken in December 1968 and diagnosis of HD firmly established. The histology was of lymphocyte-depleted type with necrosis as prominent feature. The patient was then commenced on nitrogen mustard and steroid therapy

In March 1969 the patient was readmitted complaining of nausea and vomiting of 5 days duration. Clinical examination was negative apart from oral candidiasis. I particular she had no palpable hepatosplenomegaly or peripheral lymphadenopathy Examination of the peripheral blood showed Hb 9.3 g dl, WBC, 1.3×10^9/l with a differential of 36% neutrophils, 6% eosinophils, 12% lymphocytes and 46% monocytes, including numerous typical and pleomorphic forms of 'glandular fever' type. A diagnosis of IM was strongly suspected on haematological grounds and this was confirmed by the finding f positive IM slide screening test (Monospot), diagnostic differential absorption titre of 1.256 following guinea pig kidney absorption, and an EBV antibody titre of 1 160. Serial examination revealed rising EBV and falling IM heterophil antibody titre Thi was accompanied by gradual improvement in the patient clinical condition Within 2 weeks, the atypical mononuclear cell had disappeared from the peripheral blood, and ithin 3 months, both the IM slide and differential bsorption tests for heterophil antibody had become negative.

Following recovery from this intercurrent illness, the patient HD continued to progress, and in April 1970, she died from septicaemia while receiving intensive cy totoxic therapy

Comment

This report, describing the development of IM in a patient receiving treatment for HD is in contrast to that previously reported, where IM followed its curative treatment [7]. Although unexpected false positive persisting IM heterophil antibody [1] and transient Monospot test

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Key Words. Epstein-Barr virus · Hodgkin's disease · Infectious mononucleosis

Abstract. A patient who developed infectious mononucleosis while being treated for Hodgkin's disease is presented. This pathological profile does not support the concept of a causal relationship between Epstein-Barr virus and Hodgkin's disease.

The aetiology of Hodgkin's disease (HD) is still uncertain, although several epidemiological studies have suggested an association between HD and preceeding infectious mononucleosis (IM) [3, 11, 13, 14]. While it is now widely accepted that the causal agent in IM is Epstein Barr virus (EBV) [5, 15], the role of this virus and IM in relation to HD remains controversial. Although initial studies [2, 9] showed significantly higher EBV antibody levels in patients with HD compared to controls, Langenhuysen *et al.* [8], using more sensitive laboratory techniques, subsequently demonstrated elevated antibody titres to both EBV and cytomegalovirus. Serious doubts have therefore been cast on any specific or causal role for EBV in HD. This view gains support from the few descriptions of the simultaneous presentation of the two disorders [4, 10] and even more, from the recent and previously unreported development of IM in a patient who 3 years previously had undergone curative treatment for HD [7]. The purpose of this brief communication is to report the occurrence of IM in a further patient with established HD.

We acknowledge Dr. J. F. Banatvala, St. Thomas' Hospital, London, for measurement of the Epstein-Barr virus antibody levels.

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[16] have been singularly reported in HD we feel that the typical peripheral blood picture and pattern of IM heterophil and EBV antibody titres, establish beyond doubt, the diagnosis of IM in our patient, whose clinical presentation had been uncharacteristic. The sequence of events in this case thus supports the conclusion of Langenhuysen [7] and in addition must strongly question the suggested causal relationship between EBV and HD

Finally the paucity of reports of IM occurring in established HD may be explained by the low incidence of HD in the younger age groups (in the area of this study 1/43 000 subjects aged 0–24 years at risk per annum) and by the 90% EBV seroconversion known to occur by early adulthood [6, 12]

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*were made by subjecting the polymorph fractions to ultrasound: cells were ex*posed to sound waves at frequency of 20 kcps for 30-sec periods in an ice-bath until destruction appeared complete microscopically Serum treated in this way did not lose any folate-binding power 6 age-matched female controls and 3 male controls wer studied in an exactly similar manner

The fractionation studies were carried out in Sephadex G 200 gel in a column 30×2.5 cm, 0.1 ml phosphate buffer (pH 7.4) containing 0.5 ml sodium chloride was used as eluting fluid throughout. Optical density of the 5-ml fractions was measured by ultraviolet light detector Proteins of known molecular weight including β-lactoglobulin were also passed down the column in order to calibrate it and make it possible to correlate fraction number with appr ximate molecular weight. The subjects sera and lysates nd the controls sera were incubated with tritiated PGA (^{3}H PGA) t 37 °C for 1 h before fractionation. From knowledge of the FABP *of each serum and lysat it was possible to incubate each test sample with* 100% excess of H PGA 3ml aliquots containing varying proportions of test substances and phosphate buffer were added to the column. The proportion of test substance was varied up to maximum of 1 ml in order to produce satisfactory level of radioactivity in the fractions The fractions were counted with scintillation counter

Studies were also carried out using human milk and pig serum which was fractionated in the same way at suitable dilution after incubation with ^{3}H-PGA. Counts were plotted for each fraction number on the optical density curve.

Serum and red cell folates were measured by the L-casei method and serum B_{12} by saturation analyses using ^{57}Co-cyanocobalamin.

Results

Table I shows the FABP levels of the sera and polymorph lysates together with details of each subject and control. Full blood count, serum B_{12} and serum B_{12} binders were normal in all cases except for subject S.C. who had a serum B_{12} of 90 pg/ml (normal 120–900 pg/ml) but her Schilling test was normal. Lysates of monocytes and lymphocytes consistently gave values of less than 5 pg/ml, i.e levels not measurable by the assay

A typical result of fractionation studies is shown in figure 1 The peaks of radioactivity represent respectively the bound PGA and free PGA eluted off the column with substances of molecular weight less than 10,000 This latter peak is a constant fraction in each experiment and can be reproduced by passing ^{3}-PGA alone down the column It is removed by treating the fractions with albumin-coated charcoal

Human milk (considerably diluted), however ga e two peaks in addition to the terminal one the second (major) peak occurred in the same

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A Study of Folic-Acid Binding Protein in Normal Subjects

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Key Words Chromatography Folic-acid binding protein Heparin Leucocyte lysates

Abstract 6 normal females were found to have a high level of folic-acid-binding protein (FABP) in their sera (mean level 345 pg/ml) Lysates of their various leucocytes were assayed and FABP found exclusively in the polymorph fraction. The sera and lysates were fractionated by Sephadex G-200 gel column chromatography and the molecular weight of the FABP appeared to be approximately 40,000 A hitherto unreported inhib tory effect of heparin on folate binding wa noticed.

Studies have been performed on folic-acid-binding protein (FABP) in various pathological states, but the levels found in normal people are usually considered too low to permit investigation. In an earlier study [3], 6 normal females were found to have levels in excess of 300 pg/ml whilst the mean level of 94 controls was 76 pg/ml Sera and leucocyte lysates from these people have been used to esti mate the molecular weight of FABP in normal people.

Subjects and Methods

The subjects were healthy females aged 21–65 At the time of this study none was pregnant, 1 (E.N) was taki g an oral contraceptive. 1 subject (K.M) appeared to be mildly iron-deficient, but the remainder had no haematological abnormality and, in particular there was no evidence of folate deficiency

Venous blood was taken at 8.30 a.m and either heparinised or llowed to clot in plain tubes. FABP was measured as in the previous study [3] Heparinised blood was separated into its various cell types by the method of SUMMERS *et al* [5] and FABP estimated. It proved possible to produce polymorph preparations of purity greater than 99% and after initial studies had shown all FABP to derive from the polymorph fraction, this fraction only was isolated for study Lysates

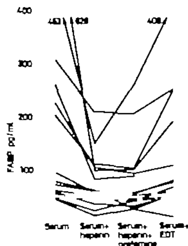


Fig. 3 Variation of FABP with anticoagulant

Table 1 Details of subjects and controls

Subject	Age	Sex	Serum FABP pg/ml	Lymph FABP pg/10- cells	Serum folate ng/ml	RBC folate ng/ml
K. M.	30–40	F	439	628	4	496
G. M.	30–40	F	270	564	3.5	327
E. N.	20–30	F	614	417	3.4	263
S. A.	20–30	F	318	251	15	287
E. R.	60+	F	225	265	18	246
L. S.	20–30	F	205	459	18	293
J. C.	20–30	F	76	5	3.1	593
D. G.	20–30	M	53	<5	3	701
M. C.	30–40	F	87	5	>18	311
M. S.	60+	F	46	<5	>18	427
P. E.	20–30	M	60	<5	3	378
T. F.	20–30	M	39	5	3	726
C. H.	20–30	F	68	5	14	351
S. C.	20–30	F	40	<5	3.5	493
A. B.	30–40	F	72	<5	>18	287
Normal					3–18	160–640

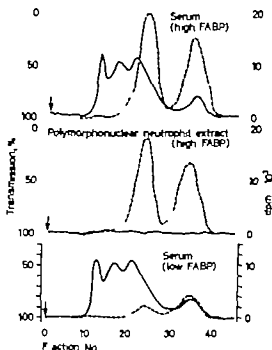


Fig. 1. Examples of fractionation (Sephadex G 200) of sera and polymorphonuclear leucocyte lysates.

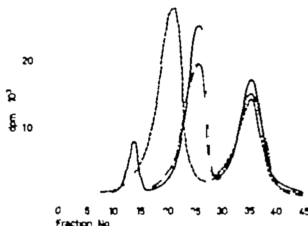


Fig. 2. Fractionation (Sephadex G-200) of human high FABP serum (———), pig serum (---) and diluted human milk (—).

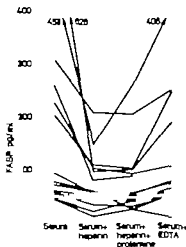


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A. B.	30–40	F	72	<5	>18	487
Normal					3–18	160–640

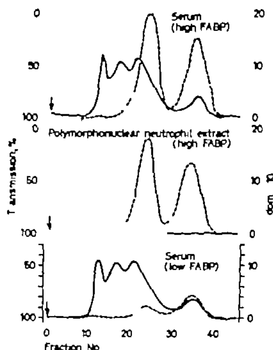


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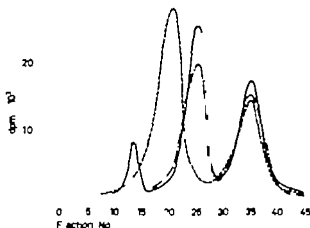


Fig. 2 Fractionation (Sephadex G-200) of human high FABP serum (-----), pig serum (—–) and diluted human milk (—).

teristics of FABP in the serum in folate deficiency [6] pregnancy [1] and some cases of chronic myeloid leukaemia [4] No significant macromolecular peak was found in normal subjects.

The heparin effect, although inconstant, is of interest if only because heparin is known to affect the elution pattern of bound B_{12} from chromatographic columns [2]

FABP is most likely an intracellular protein involved perhaps in regulation of folate absorption, or in controlling the availability of folate coenzyme in DNA synthesis. Circulating FABP in the serum probably is only a reflection of intracellular binder

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fraction as the binding peak in normal serum The chromatographic pattern seen in human milk is in accord with the findings of others [7] Pig serum gave a single peak with a molecular weight higher than that found in normal human serum (fig. 2)

Heparin effect On one occasion heparinised plasma was used to assay FABP in place of serum from a subject known to have a high level. A low result was obtained The expected value was partly regained after neutralisation with protamine. Sequestrene (EDTA) did not have such an effect and it did not apply to all FABP – for example no heparin effect was noticed on serum from a patient with smouldering acute myeloid leukaemia and a high FABP level In normal subjects with low FABP very little effect was noticed (fig 3)

Discussion

By examining those subjects at the uppermost end of our group of normals, it was possible to investigate the nature of FABP in the serum and leucocytes of normal people These people may represent one end of a normal spectrum as none of the factors known to produce a high FABP were present with the exception of E.N who was taking oral contraceptives at the time of this study She is included because she was found to have a high level before the drug was started 3 of the control subjects were also taking oral contraceptives

Levels of FABP were found in the polymorphonuclear neutrophils parallel to those found in the serum and tended to remain fairly constant in the same subjects on different occasions. Measurable FABP was not found in any other leucocyte lysate than the neutrophil This contradicts our earlier idea that the monocyte might be a source of FABP but the situation in leukaemia is rather different. It seems likely that the distribution of folate binders in leukaemia is not the same as that found in normal people fractionation studies of serum from a leukaemic patient showed a different pattern and blast cell lysates from 3 cases of myeloid leukaemia showed negligible folate binding power Further studies are in progress.

Fractionation of sera and lysates in every case has shown that the binder elutes from the column in the same fraction as β lactoglobulin and therefore, has an apparent molecular weight of approximately 40 000 This is in accordance with other published work on the charac

were sequestered by the spleen [1, 2]. There is some evidence that Heinz bodies become bound to the inside of the red cell membrane of patients with unstable hemoglobins. This results in an increased red cell rigidity [3]. Splenectomy is of benefit to these patients when hemolysis is so excessive that blood transfusions become necessary. On the other hand, splenectomy rarely, if ever, benefits patients with chronic hemolysis due to deficiencies in the enzymes of the hexose monophosphate pathway [4]. A very low enzymatic activity of the glucose-6-phosphate dehydrogenase variant (G-6-PD Hamburg) was reported to occur in erythrocytes of a patient with chronic nonspherocytic hemolytic anemia [5, 6]. The present paper describes a study of the deformability of red cells and the enzymatic activity of G-6-PD Hamburg in ghosts of erythrocytes from this patient with hemolytic anemia.

Material and Methods

Subjects. Blood samples from 24 healthy adults were used as controls. Whole red cell populations from eight adults were separated into populations of young and old cells by centrifugation (30,000 *g* for 30 min) using the method of OSKI *et al.* [7]. A volume of 10% of the whole fraction was carefully aspirated from both the top and bottom of the tube. Erythrocyte suspensions of hemolysates and ghosts were prepared from these samples. Reticulocytes were counted in the top and bottom fractions to determine the mean age of each cell population [7, 8]. Two blood samples were collected from the patient within 6 weeks. Erythrocyte suspensions, hemolysates, and ghosts were prepared from these samples. Coagulation was prevented by the addition of 7 IU of preservative-free heparin per milliliter blood. The hematological data for the patient and the biochemical characteristics of the enzyme variant were reported previously [5, 6].

Filtration studies. The filtration method described by SCHMID-SCHÖNBEIN *et al.* [9] was used to measure red cell deformability. The buffy coat was removed and the plasma was aspirated after centrifugation of the samples. The flow rates of plasma and of erythrocyte suspensions were determined by measuring the times required for the complete passage of 1 ml through polycarbonate sieves with a nominal pore diameter of 5 μm (Type Nucleopore, Thomas Comp., Philadelphia, Pa.). The flow rates of both the controls and of the patient were also determined from erythrocyte suspensions with and without acetylphenylhydrazine incubation.

Heinz body formation. Heinz body formation in erythrocytes was determined in blood samples of the patient and of eight healthy adults. 3 ml of washed, packed red cells were resuspended in 6 ml of a buffer medium containing three parts isotonic saline and one part 0.1 M Sorensen phosphate buffer, pH 7.4. Acetylphenylhydrazine was added to the buffer medium to give a final concentration of 13 mmol/l. During incubation for 195 min the suspensions were slightly agitated (300 oscillations/h). Aliquots of the suspensions were removed to count the num-

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Membrane Deformability of Erythrocytes with Glucose-6-Phosphate Dehydrogenase Hamburg[1]

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Key Words. Enzyme erythropathies Ghost enzymes G-6-PD Hamburg Heinz bodies Hemolytic anemia Red cell deformability Red cell membrane Splenectomy

Abstract Deformability of erythrocytes of a patient with chronic nonspherocytic hemolytic anemia caused by a G-6-PD variant (G-6-PD Hamburg) in red cells was studied. The flow rate of erythrocytes from this patient through 5 μm polycarbonate sieves was higher than that of red cells from healthy adults. Even under oxidative stress *in vitro*, the deformability of erythrocytes of the patient was only slightly decreased The residual hemoglobin content of red cell membranes (ghosts) from this patient was lower than that of ghosts from healthy adults and of ghosts prepared from comparable reticulocyte-rich blood without G-6-PD deficiency In contrast to the low enzymatic activity of G-6-PD Hamburg in the hemolysate, a high activity of this enzyme could be demonstrated in the ghosts from the patient. In view of the flexibility of this patient's erythrocytes splenectomy is not to be recommended.

Glucose-6-phosphate dehydrogenase (G-6-PD) plays an important role by protecting SH groups of the red cell against oxidative stress by generating NADPH to maintain glutathione in the reduced form. Increased oxidative stress due to drugs or infections leads to an increased hemolysis of red cells of patients with G-6-PD deficiency *in vivo* When these cells are stressed *in vitro* by incubation with acetylphenylhydrazine an excessive number of Heinz bodies may be seen Rifkind showed by electron micrographic studies that red cells containing Heinz bodies

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Table 1. Flow rate of erythrocytes, hemoglobin content of ghosts and enzyme activities in hemolysates and ghosts

	Whole population of adults	Young cells of adults	Old cells of adults	Patient
Flow rate of erythrocytes, µl/sec	54.31 ± 4.02 (n = 4)	65.48 ± 4.71 (n = 8)	51.51 ± 4.90 (n = 8)	66.56
Flow rate of incubated erythrocytes containing Heinz bodies, µl/sec	13.91 ± 2.35 (n = 8)			51.28[a]
Hemoglobin content of ghosts, mg/10 ghosts	32.00 ± 4.10 (n = 18)	77.2 ± 0.32 (= 8)	37.9 ± 0.31 (= 8)	20.60
Activity of G-6-PD				
in hemolysate, IU/10[3] erythrocytes	30.60 ± 4.50 (= 36)			0.20
in ghosts, IU/10 ghosts	0.30 ± 0.08 (= 4)	0.43 ± 0.11 (n = 8)	0.25 ± 0.08 (n = 8)	0.55
Activity of hexokinase				
in hemolysates, IU/10 erythrocytes	2.33 ± 0.45 (= 4)			4.93
in ghosts, IU/10[3] ghosts	0.13 ± 0.04 (= 24)	0.15 ± 0.01 (n = 8)	0.10 ± 0.03 (n = 8)	0.77
Reticulocytes, %	0.90 ± 0.40 (= 4)	4.50 ± 0.80 (= 8)	0.10 ± 0.05 (n = 8)	20.30

Values of the patient are the mean of two experiments, all other values are expressed as the mean ± SD

All erythrocytes of adults contained four or more small Heinz bodies after incubation with acetylphenylhydrazine

All erythrocytes of the patient contained six or more small and large Heinz bodies.

Erythrocytes of controls and of the patient contained Heinz bodies after incubation with acetylphenylhydrazine. The number and size of these Heinz bodies was greater in the erythrocytes of the patient. The flow rate of erythrocytes from adults containing Heinz bodies was lower than from those which had not been incubated. Also, the flow rate of the incubated erythrocytes of the patient was lower than that of the non-incubated cells of the patient but not as much as the flow rate of erythrocytes of adults. As well as an increased amount of hemoglobin at

ber of erythrocytes containing Heinz bodies or to determine flow rates. After staining with Nilbluesulfate the percentage of Heinz body containing erythrocytes was determined by means of duplicate 1,000 cell counts.

Preparation of ghosts. Isolated red cell membranes (ghosts) were prepared by modifications of the method of DODGE *et al.* [10, 11]. The washed cells were lysed three times in a hypotonic 30 mosm phosphate buffer solution (pH 7.4) at 4 °C. The resulting ghosts were intact as viewed by phase-contrast microscopy. Each batch of ghosts was divided into two parts, either for measurement of hemoglobin content, or for determining of enzyme activity. The hemoglobin content of the ghosts was measured by the method described by DACIE and LEWIS [12].

Assay of enzyme activity in hemolysates and ghosts. Activity of G-6-PD in hemolysates was determined at 37 °C, using the WHO method [WHO scientific group 1967] as described earlier [5]. For measuring enzymatic activity in ghost suspensions, glucose-6-phosphate, as substrate, was added in a concentration of 6×10^{-3} M. Assay of hexokinase activity was carried out as described before [11]. All assays were carried out in duplicate and appropriate blanks were used. Enzyme units were defined as the number of μmoles of pyridine nucleotide converted per minute at 37 °C per 10^{11} erythrocytes, or per 10^{11} ghosts, respectively.

Results and Discussion

Ghosts of young erythrocytes retained less hemoglobin than the ghosts of older cells (table I). The ghosts from the patient had the lowest hemoglobin content. The mean age of the patient's erythrocytes was even lower than that of the young erythrocyte fractions from the normal controls. This was demonstrated by the higher reticulocyte count. WEED *et al.* [13] have demonstrated that red cell deformability depends on the amount of hemoglobin attached to the membrane. Table I shows that the flow rate and thus the deformability of young cells was higher than that of the older ones, while the erythrocytes of the patient had the highest deformability of all. We suggest that the high deformability of the erythrocytes of the patient is related to the fact that these cells are younger. By contrast, erythrocytes of patients with an unstable hemoglobin (hemoglobin Köln) have more than normal amounts of hemoglobin attached to their membranes, low flow rates, and thus less than normal deformability [14]. The decreased deformability of erythrocytes, measurable *in vitro*, corresponds to an increased destruction of the cells in the spleen of patients with Hb Köln. It is suggested that the amount of hemoglobin attached to the membrane plays an important role in the decreased deformability of these red cells and that the decreased deformability may reduce the longevity of these red cells.

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tached to the membrane the presence of Heinz bodies in the erythrocytes diminishes the flexibility of the cell as earlier described by LUBIN and DESFORGES [15] and TILLMANN *et al* [14] The only slightly decreased flexibility of the Heinz body containing erythrocytes of the patient is explained by the diminished attachment of hemoglobin to the erythrocyte membranes of the patient.

From these results we conclude that the spleen does not play an important role in the destruction of erythrocytes with G-6-PD Hamburg. Even under oxidative stress the spleen may not destroy a greater number of the patient's red cells. This is suggested by the only slightly decreased flow rate of the erythrocytes after incubation with acetylphenyl hydrazine Study of other forms of G-6-PD deficiency may show that erythrocyte deformability is unaltered in contrast to patients with unstable hemoglobin Köln Enzymatic activity of G-6-PD determined in hemolysates of the patient, was diminished to 0.2 IU/10^{11} erythrocytes as previously reported [5] Table I shows that about 1% of G-6-PD activity measured in hemolysates was detected in ghost suspensions of healthy adults prepared by a hypotonic 30 mosm phosphate buffer as previously demonstrated [11] With regard to the low activity of the G-6-PD variant in the hemolysate of the patient, an unexpectedly high value of enzymatic activity was determined in the ghost suspension of the patient. Also the activity of hexokinase was higher in ghost suspensions from the patient than in ghosts of healthy adults The high activity of this enzyme correlates well with the reticulocytosis of the patient but it is not yet explained why the G-6-PD activity was high in ghosts of the patient The reticulocytosis cannot explain this fact alone In ghosts, prepared from blood without enzyme deficiency with comparatively high reticulocyte counts, G-6-PD activity amounts only 0 43 IU/10^{11} ghosts. Experiments with partially purified G-6-PD Hamburg could be interpreted to mean that this variant enzyme has an increased tendency to exist in the monomeric, catalytically inactive state [16] Attachment of this variant to the red cell membrane could stabilize the dimeric state, which is catalytically active in the normal enzyme, but this explanation is only speculative

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Table I Plasma calcium determination in normals and May-Hegglin anomaly

	Platelet count/µl	Platelet calcium ng/200,000 platelets	Plasma calcium mg %
Normal	150,000–400,000	12 ± 3	8.8–10.8
May-Hegglin			
Case 1	115,000	25.-	8.8
Case 2	87,000	16.5	9.0
Case 3	68,000	51.0	9.0
Case 4	80,000	41.25	9.1
Case 5	63,000	53.0	8.9

was used also from the initial PRP. The two tubes were assayed in the step two for their calcium concentration by atomic absorption spectrophotometry in Perkin-Elmer model 290 B instrument with a dual lamp for calcium-magnesium fixed at 457 nm. Monitrol was used as standard at concentration of 10 mg and fixed on the scale of the spectrophotometer at the 50th division. Care must be taken to use only plastic tubes, pipettes or glass-siliconized instruments, in order to avoid platelets' adhesivity.

The platelet count in PRP was done on light microscope using an improved Neubauer chamber [3] and the result was given in platelets per microliter. The initial dilution of PRP with ADP was taken into account in the final result as well as the difference between platelet-poor plasma calcium and platelet-poor plasma calcium with added ADP. We found that they do not exceed 1 mg/100 ml, so that the formula used was: read PRP – read PRP release × 1 = platelet calcium/mg/ml. This final result was brought for standardization reasons to ng/200,000 platelets.

Results and Discussion

In our determinations of calcium in platelets by atomic absorption spectrophotometry we found higher values in May Hegglin anomaly than in the normals, while the plasma calcium was in all cases within the normal range (table I). A number of investigators suggested that calcium may be the important factor that governs the activity of the contractile material. We found this explanation in agreement with our high values in divalent cations in the platelets of the May-Hegglin disorder and tried to explain why the clot retraction is unaffected as in other thrombocytopenic states.

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A Simple and Rapid Platelet Calcium Determination

Normal Values and Results in May-Hegglin Anomaly

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Key Words. Atomic absorption spectrophotometry Calcium in thrombocytes May Hegglin anomaly Platelet calcium determination

Abstract A simple and rapid method for platelet calcium determination by atomic absorption spectrophotometry is described. The results in normals and in May Hegglin anomaly are given.

During the last years large studies in the physiology ultrastructure and biochemistry of platelets were performed [1] The examinations include hemostatic functions, enzymes, protein synthesis and calcium magnesium determinations

We describe a simple and rapid method for platelet calcium determination using the atomic absorption spectrophotometry technique

Materials and Methods

20 healthy volunteers were investigated in comparison with a family affected by May-Hegglin anomaly [2] for their calcium concentration in plasma and platelets. 10 ml of blood were withdrawn in Na citrate 3.8 g‰ as anticoagulant in a proportion of 1/9 and from this, platelet rich plasma (PRP) was obtained by centrifugation at 85 g for 10 min.

Platelet calcium was determined by a two-step method. To 0.6 ml PRP was added 0.2 ml ADP 1 μg/ml and the reaction allowed to proceed for 6 min with continuous mixing at 37 °C. At the end of this time the plastic tubes were frozen and thawed three times and spunned for 30 sec in an Eppendorf centrifuge (Brinkman Instruments, New York, N Y) and the supernatant was carefully separated. From this supernatant a dilution 1/10 in distilled water was done and the same dilution

Table I Plasma calcium determination in normals and May-Hegglin anomaly

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Results and Discussion

In our determinations of calcium in platelets by atomic absorption spectrophotometry we found higher values in May Hegglin anomaly than in the normals, while the plasma calcium was in all cases within the normal range (table I). A number of investigators suggested that calcium may be the important factor that governs the activity of the contractile material. We found this explanation in agreement with our high values in divalent cations in the platelets of the May Hegglin disorder and tried to explain why the clot retraction is unaffected as in other thrombocytopenic states.

The purpose of this study was to try other parameters less investigated than the hemostatic properties or ultrastructure in this anomaly and together with this to find a simple and rapid method for calcium determination in platelets. The results are in a good proportional relation with the determinations of other investigators [5] who gave their platelet calcium magnesium results in milligram per dry weight.

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Chronic Idiopathic Myelofibrosis. A Reversible Disease?

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Key Words. Angio-immunoblastic lymphadenopathy · Cytostatic therapy in myelofibrosis · Myelofibrosis reversibility · Splenectomy in myelofibrosis

Abstract. A patient with chronic idiopathic myelofibrosis was subjected to splenectomy 1 year after diagnosis. As clinically unexpected finding, lymph node biopsy suggested the presence of non-Hodgkin lymphoma. The patient was subjected to intensive combined cytostatic therapy. I the following months, signs and symptoms f myelofibrosis regressed remarkably. The patient died 31 months after splenectomy in massive gastrointestinal bleeding. At post-mortem, myelofibrosis could not be detected in three bone marrow areas and regular fat-containing, hypercellular marrow was present. The nature of the previous lymph node pathology was reconsidered, and angioimmunoblastic lymphadenopathy was diagnosed.

In 1958 Botos *et al.* [1] published a case (male, 43 years old) of chronic idiopathic myelofibrosis (CIM) in which trephine biopsy (made in 1952) proved marked diffuse myelofibrosis. Panhaemopoiesis was present in the histological sections of liver and spleen biopsies and signs of myeloid metaplasia were demonstrable in the circulating blood. In January 1956, splenectomy has been performed, marked extramedullary haemopoiesis was demonstrated again. In August 1956 a new trephine biopsy showed haemopoietic areas along with myelofibrosis. In October 1956 the patient died of pulmonary embolism. The histological pattern of four different bone marrow regions showed only a slight myelofibrosis; compared to earlier preparates a remarkable regression of fibrosis was demonstrable. Only a few haemopoietic cells were present in liver sections. The possible reversibility of CIM was considered [4].

Case Report

Recently a more pronounced regression of CIM occurred in a 61 year-old woman. At the first admission in April 1972 the patient had the classic signs and symptoms of CIM with a splenomegaly reaching the iliac crest, with histologically demonstrated myeloid metaplasia in spleen and liver and with appropriate cellular pattern in the circulating blood No previous haematological or other disease could be detected. Some granulocyte precursors were present in peripheral blood and the number of tear-drop cells amounted to 120/5 000 RBC. Scattered normoblasts could be detected and several macrothrombocytes along with occasional megakaryocyte nuclei were found. Trephine biopsy proved remarkable marrow fibrosis with preserved areas of haemopoietic cells.

She had 9.5 g Hb/dl 25 × 10^9/l reticulocytes, 66 × 10^9/l granulocytes and 120 × 10^9/l platelets. Conventional treatment with androgenic steroid and folic acid was applied for months, but even the Hb value failed to improve. In the course of the 1st year of our observation the maximum granulocyte count was 11.8 × 10^9/l and the platelet counts ranged between 100 and 140 × 10^9/l blood She was readmitted in April 1973 Blood cell counts and morphology did not change substantially but the size of the spleen increased remarkably Occasional myeloblasts were noted in blood smears and the neutrophil alkaline phosphatase score was 350. Temperature was normal

In May 1973 splenectomy was performed (Prof A SZECSÉNYI) The spleen weighed 4500 g (50 kg body weight) Iliac crest and liver biopsies were performed again, and a small splenic hilar lymph gland was investigated too. There was a diffuse (van Gieson-positive) fibrosis with virtual absence of haemopoietic areas and fat cells in the marrow (fig. 1), along with pronounced increase in trabecular fibre network (Gömöri's silver impregnation) (fig.) with osteoid formation as well as with occasional lamellar trabeculae Panhaemopoietic metaplasia was present in the spleen (fig. 3) and liver Apart from this, although the lymphoid cell representation of the spleen was practically restricted to Malpighian bodies and areas of atrophy and fibrosis could be found in it there were some proliferating lymphoid cells in the liver and there was (a) a diffuse alteration of the nodal structure (b) an intensive lymphoproliferation along with various cells of a stimulated lymphoid apparatus, including immunoblasts with occasional haemopoietic clusters, and (c) a prominent vascular proliferation in the small lymph gland (fig. 4) In July 1973 a soybean-sized axillary lymph gland has been excised. Its histology was identical with the former one, and owing to the pronounced diffuse lymphoid proliferation with several mitoses that caused the disappearance of lymph gland structure and was demonstrable even pericapsularly a malignant non Hodgkin lymphoma was (erroneously) diagnosed.

Combined cytostatic chemotherapy was started on the base of the well-known MOPP scheme (nitrogen mustard, vincristine procarbazine and prednisolone) Eleven courses were given from August 1973 to March 1975 seven of them in the first 9 months of therapy No dose reduction was necessary throughout this period, i e the advised doses were applied in each course [ ] Although the Hb value tended to diminish after each course, its minimum was 8 3 g/dl On the other hand, maximum value reached 17 g dl after the first nine courses. There was a pronounced granulo-

1



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Fig. 1 Diffuse myelofibrosis ith osteoid formation in the iliac crest just before splenectomy. HE. 100.

Fig. Pronounced increase in marrow fibre network (just before splenectomy and cytostatic therapy). Gomori's silver impregnation. 100.

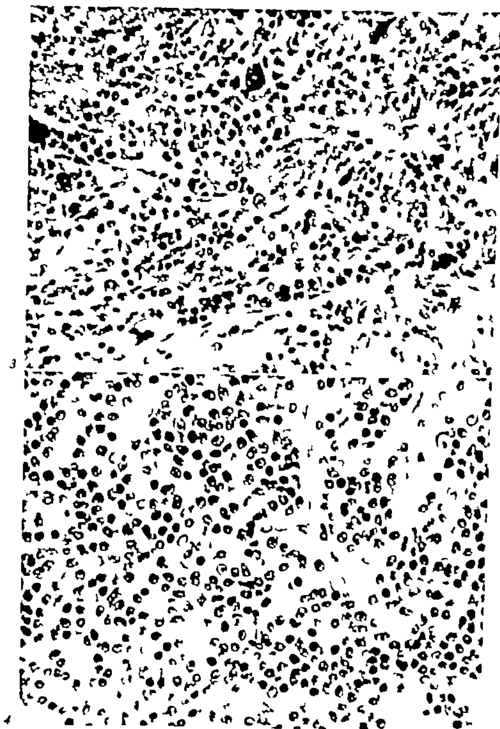


Fig 3 Myeloid metaplasia in the spleen (at splenectomy). HE. × 300.

Fig 4 Angioimmunoblastic lymphadenopathy (at splenectomy see text) HE. × 300.

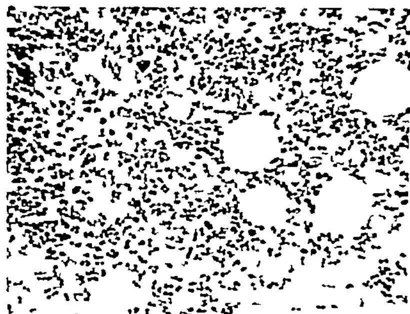


Fig 5 Postmortem femoral bone marrow Increased cellularity with various precursors and ordinary f t. Myelofibrosis is absent. HE. 150.

cytosis befor each new course and its maximum reached 31 × 10^9/l. The participation of eosinophils and basophils was recognizable after splenectomy and the number of circulating normoblasts was increased. After splenectomy the platelet count rose to 1,900 × 10^9/l (3–4 months after splenectomy), but remained under 800 × 10^9/l during the nine MOPP courses. Despite several dry taps t the start of the disease process, as well as once after splenectomy in December 1974 normocellular marrow was aspirated which exhibited a practically normal cell composition. Philadelphia chromosome was not found.

Although even the eleventh course of combined cytostatic therapy (March 1975) reduced the circulating granulocytes from 27.5 to 6.7 × 10^9/l, the platelet count e-mained at 800 × 10^9/l. The Hb level which was 12.3 g/dl at the start of this course was 9.7 g/dl after it, and unlike after previous courses, it diminished to 7.7 g/dl at June 1975 along with an levation of circulating platelet count to 1,500 × 10^9/l. At this stage we abandoned MOPP therapy and two 3-day courses of mitobronitol (Myelobromol) have been applied with 80 days between the two courses. Each course consisted of 4–3–2 tablets (250 mg/tablet), 2,250 mg altogether in course. The Hb level rose stepwise and eached 17.4 g dl in November 1975. She had 110 × 10^9/l reticulocytes. The number of granulocytes fluctuated from 27.6 to 9.5 × 10^9/l during the first, and from 21.3 to 8 × 10^9/l during the second Myelo-

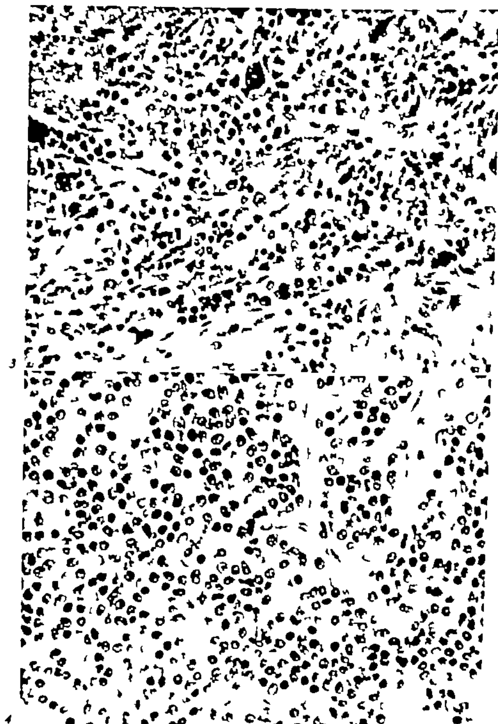


Fig 3 Myeloid metaplasia in the spleen (at splenectomy). HE. × 300.

Fig 4 Angioimmunoblastic lymphadenopathy (at splenectomy see text) HE. × 300.

with splenectomy alone in CIM, among these reports of fairly good clinical improvements [7] but reversibility of the process has not been considered.

Recently we started to treat a few other CIM patients similarly but *owing to the fact that many months elapse before any beneficial effect of* this therapy should be ascertained, we decided to publish the present preliminary report. Of course, premature generalization must be avoided.

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bromol course. Circulating platelets diminished to 250 × 10⁹/l during the first course, reached 820 × 10⁹/l before the second one, and were at 300–400 × 10⁹/l after the second course. Although the number of band forms reached 20%, circulating immature granulocytes and normoblasts were scanty. There were only three tear drop cells among 5 000 RBC. Giant platelets could be detected.

At the last month of 1975 she was admitted to a county hospital with herpes zoster and developed gastrointestinal bleeding (430 × 10⁹/l platelets) followed by massive haematemesis and melaena originating from multiple exulcerating gastric erosions. She was subjected to surgical intervention and died in December 1975 in haemorrhagic shock.

At autopsy three bone marrow areas showed virtual absence of fibrosis: a regular rather left-shifted hypercellular marrow picture could be seen, with round-oval fatty spots (fig. 5). All haemopoietic systems were represented. Lymphocytes and plasma cells were present in normal number. The formerly abundant reticular fibre network as well as pathological collagen fibres were no more apparent (courtesy of Dr. G. LUSZTIG, Kecskemét). There was no myeloid metaplasia in the liver and in the lymph nodes. The nodes exhibited recognizable structure along with lymphoid depletion and intensive histiocytic and macrophage proliferation.

The re-evaluation of the older (1973) lymph nodes in three different institutes showed that the histological picture was erroneously classified as malignant lymphoma, and accords to angioimmunoblastic lymphadenopathy [3]. Although the corticomedullary lymphoid hyperplasia blurred the lymph gland structure, the lymphoid cells were morphologically heterogeneous and a pronounced proliferation of blood vessels was present. In addition, there was no sign of lymphoid malignancy in the big spleen. The fact that, sometimes, a malignant lymphoreticular process could be simulated in CIM is mentioned in the monograph of STORTI and PERUGINI [7].

Discussion

So far this is our second case in which the reversibility of CIM became apparent. Well known recent haematology textbooks do not mention this possibility. In 1975 an apparently similar reversibility was, however, observed in a case of CIM subjected to splenectomy [6]. In this case, too, malignant lymphoma has been considered, but was not proved subsequently. No autopsy was obtained. Further, MYERS *et al.* [5] reported that bone marrow involvement in Hodgkin's disease can be safely and effectively treated with intensive chemotherapy and pathological changes in the marrow, including diffuse myelofibrosis, are often reversible with such therapy.

The relative significance of splenectomy, MOPP therapy, mitobronitol or any other factor in this surprising, apparently subtotal reversibility cannot be evaluated at present. In any event, there is sufficient experience

teins [12] and lymphocytes [18] which are quickly withdrawn from the circulating blood.

On the basis of the above considerations, in an attempt to elucidate the mechanism by which erythrocyte membrane is modified during physiological aging, the study of the fate *in vivo* of rabbit erythrocytes after neuraminidase removal of different amounts of sialic acid was undertaken. This investigation was carried out using both total erythrocyte populations and reticulocyte-enriched blood.

Methods

Fauve de Bourgogne rabbit (about 3 kg) were used for the experiments.

Neuraminidase treatment. *Clostridium perfringens* neuraminidase was obtained from Sigma (type VI). 1 unit of the enzyme will liberate 1 µmole of N-acetylneuraminic acid per minute at pH 5 and 37 °C, using either N-acetylneuraminolactose or bovine submaxillary mucin as substrate. For each experiment 12 ml of blood were drawn from the heart of the rabbit by heparinized syringe. After 3 washes with 0.9% NaCl, packed erythrocytes were suspended in the same solution so as to reach haematocrit level of about 40%. One third of the sample was used for the determination of the ghost sialic acid content (see below). One third was incubated for 10 min at 37 °C with different amounts of neuraminidase (from 0.005 to 0.16 U/1 ml of the incubation mixture) under gentle shaking. Control incubations were carried out without the enzyme and with heat-inactivated neuraminidase. The last third of the sample was incubated with the same amounts of neuraminidase and with $Na_2{}^{51}CrO_4$ (30 µCi) in the same conditions as reported above, and was utilized for erythrokinetic studies. $Na_2{}^{51}CrO_4$ (specific activity 153 mCi/mg) was obtained from Sorin.

Preparation of the ghosts. After removal of excess neuraminidase, when the enzyme was present, erythrocytes were lysed and ghosts prepared as previously reported [2].

Sialic acid assay. The ghosts were hydrolyzed in 0.1 N H_2SO_4 for 1 h at 80 °C; sialic acid was then assayed in the supernatant by the method of Aminoff [1].

Survival time of ⁵¹Cr-labelled intact and neuraminidase-treated erythrocytes. After incubation with ⁵¹Cr the erythrocytes were washed twice with 0.9% NaCl and then reinjected into the marginal ear vein of the same rabbit. Blood samples (1 ml) were collected at various time intervals and radioactivity was determined both using the total sample and washed cells by Packard Tri-Carb Scintillation Spectrometer model 2002. $T_{1/2}$ value was obtained by extrapolation.

Liver and spleen radioactivity. The radioactivity present in liver and spleen was determined, after sacrificing the animal, on several pieces of tissue which were taken away from the central part of the liver and spleen. Each piece of tissue weighed about 500 mg. The determinations were carried out when blood radioactivity reached the T/50 values. In some cases when neuraminidase treatment induced

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In vivo Behaviour of Neuraminidase-Treated Rabbit Erythrocytes and Reticulocytes

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Key Words Erythrocyte life-span Erythrocyte membrane Neuraminidase Rabbit erythrocytes Sialic acid

Abstract ^{51}Cr rabbit erythrocytes were treated with different amounts of neuraminidase and reinjected into the animal. The survival curves after the removal of more than 50% membrane sialic acid show a characteristic behaviour after a rapid decrease blood radioactivity increases again reaching a maximum level 50–80 h after reinjection, then tends to decrease with a slope similar to that of control curves. Liver radioactivity determined before the rise of blood radioactivity is evidently higher than the value determined after radioactivity elevation. Similar results were obtained with phenylhydrazine-induced young erythrocytes.

The glycoproteins located at the external surface of the cell membrane play a very important biological role since they can act as antigenic determinants and virus receptor sites, and are involved in several phenomena such as contact inhibition cell-cell interaction and neoplastic transformation [4 7 17] Moreover it has been proved that the carbohydrate chains of red cell membrane, during *in vivo* aging of the cell undergo structural modifications which mainly consist in a decrease of surface sialic acid [2, 3] The role of red cell membrane sialic acid as a determinant of the survival time *in vivo* has been recently investigated [6 8–10] it was proved that the removal of sialic acid from the external surface sharply reduces rabbit erythrocyte survival time though this component is not the only factor involved in this phenomenon [9] The behaviour of desialylated rabbit erythrocytes recalls that of neuraminidase-treated plasma glycopro-

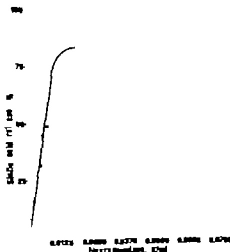


Fig. 1 Sialic acid released from rabbit erythrocyte ghosts by different amounts of neuraminidase.

Hours after injection

T/2 h

Fig. 2 Disappearance from the circulation of ^{51}Cr-labelled rabbit erythrocytes after neuraminidase removal of different amounts of sialic acid.

very quick decrease of blood radioactivity the animals were killed when blood radioactivity reached the T/20 value.

Induction of reticulocytosis by phenylhydrazine Reticulocytosis was induced in the rabbit by a daily subcutaneous injection of 1 ml of 2.5% phenylhydrazine in 0.1 M Na phosphate buffer pH 7.4 for 4 days [13] 6 days after the beginning of the treatment 90–95% of the red cells were found to be reticulocytes. Our experiments were started 8 days after the first injection of phenylhydrazine, when there were about 50-70% reticulocytes and when the haematocrit value was nearly normal The experiments with young erythrocytes were carried out with the same techniques as described previously

Separation of old cells from young cells The method of SIMON and TOPPER [15], based on the different osmotic fragility of old and young erythrocytes, was used with the same procedure as reported previously [2] Dry ghosts were assayed for their sialic acid content [1]

Determination of osmotic fragility curve The osmotic fragility curve of intact and neuraminidase-treated erythrocytes was tested according to the method of DACIE and LEWIS [5].

Results

Sialic acid content of old and young ghosts In agreement with the results of other authors [3] and with our evidence obtained with human erythrocytes, sialic acid sharply decreases in the ghosts of old erythrocytes The values (means ± SD of 3 experiments) were in young erythrocytes 0 187 ± 0 028 g/100 g and in old erythrocytes 0 111 ± 0 029 g/100 g

Erythrokinetic studies of intact and neuraminidase treated erythrocytes Red cells treated with increasing amounts of neuraminidase undergo a progressive decrease of membrane sialic acid. The released sialic acid does not exceed in any case 80–85% compared with the untreated cells, even when the neuraminidase concentration rises over 0 1 U/ml of the incubation mixture (fig. 1) Control incubations without the enzyme or with heat-inactivated enzyme induced a negligible removal of sialic acid. In agreement with literature, also in our experiments neuraminidase treatment does not modify the osmotic fragility curve [8] In our experimental conditions the T/50 value of intact and control erythrocytes ranges between 210 and 290 h.

The removal of increasing amounts of sialic acid causes a progressive decrease of T/50 which reaches a value of a few hours (5–18 h) when more than 45% of sialic acid is released from the membrane. In these conditions the ^{51}Cr survival curves become biphasic just after the reinjection of the labelled erythrocytes, blood radioactivity quickly decreases,

Table I. Erythrokinetics of intact and neuraminidase-treated rabbit red cells: spleen and liver radioactivity at T/50

Sialic acid release, %	T/50, h	Liver radioactivity	Spleen/liver radioactivity
0	293	1.7	44.8
14	135	2.4	28.2
32	125	2.6	24.6

Liver radioactivity is expressed as percentage of the total injected radioactivity.

dioactivity reached the T/50 value. In table I total liver radioactivity and spleen/liver ratio observed in erythrokinetic studies of red cells after removal of less than 45 % sialic acid are reported. It can be observed that the liver radioactivity in these experiments is only slightly increased with respect to the controls.

In some cases in which the T/50 value was very low spleen and liver radioactivity was determined when blood radioactivity reached the T/20 value, after the 'hump' (table IIa). It is quite evident that the value of liver radioactivity a few hours after reinjection and before the rise of blood radioactivity is much higher than the value determined after radioactivity transient elevation and that spleen/liver ratio is lower before than after 'hump'.

Erythrokinetic studies of intact and neuraminidase-treated rabbit reticulocytes. The experiments carried out after phenylhydrazine induction of a massive reticulocytosis were similar to those reported previously. Neuraminidase removes amounts of sialic acid from young erythrocyte membrane similar to those removed from a normal erythrocyte population. Erythrokinetic experiments proved that the T/50 value of intact young red cells was about 300 h.

After removal of 70–80% sialic acid, the ^{51}Cr erythrocyte survival curves show a biphasic behaviour resulting from an initial rapid decrease of blood radioactivity with a T/50 value of 20–25 h, followed by a rise which reaches its maximum level 100–120 h after injection and then by a new decrease with a slope similar to that of control curves (fig. 4). The percentage radioactivity which returns in peripheral blood in these experiments is slightly higher than that observed in normal erythrocyte populations. The ratio between spleen and liver radioactivity is very low when

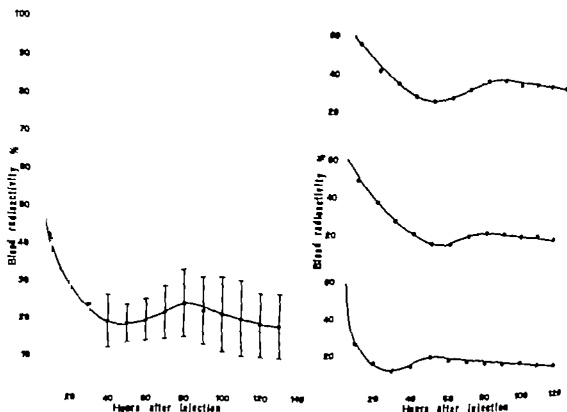


Fig 3 Blood radioactivity after reinjection of neuraminidase treated ⁵¹Cr erythrocytes (sialic acid removed 70–82%) *a* Values are means ± *SD* of 3 experiments. *b* Values obtained in each experiment.

then the curves show a much less steep slope which is similar to that of the control experiments (fig 2)

A more detailed investigation on these survival curves, carried out by taking blood samples at shorter intervals of time showed that the decrease of blood radioactivity is not simply biphasic, but behaves as reported in figure 3 After having reached a minimum value near the 30th to 50th hour after reinjection radioactivity increases until about the 58th hour and then again decreases with a slope similar to that of control curves. It seems that a portion of neuraminidase-treated erythrocytes is trapped just after reinjection in some site and then after a certain period recirculates and behaves like intact red cells. The sites where intact and enzyme-treated erythrocytes are trapped were investigated and spleen and liver radioactivity was determined after killing the rabbits when blood ra

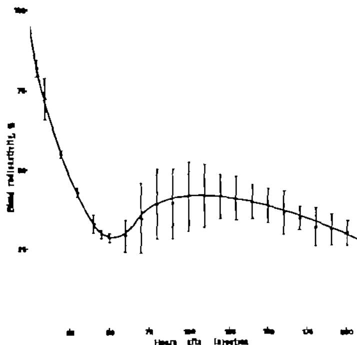


Fig. 4. Blood radioactivity after reinjection of neuraminidase-treated young ^{51}Cr rabbit erythrocytes (sialic acid removed: 75–83%). Values are means ± SD of 3 experiments.

cells and is absent in plasma, thus it can be excluded that the rise is due to the release of catabolic fragments from the reticuloendothelial system. This result suggests that a portion of neuraminidase-treated erythrocytes is rapidly withdrawn from the circulation after injection and then reappears in the blood. The slope of the survival curve after the radioactivity increase seems to indicate that erythrocytes, returned in circulating blood, behave like untreated cells. This could mean that the erythrocytes reappear in the blood when their membrane has been repaired. This phenomenon is not surprising, being reminiscent of the behaviour of neuraminidase-treated lymphocytes [18].

As far as the site of erythrocyte sequestration is concerned, the value of the ratio between spleen and liver radioactivity is low when determined before transient radioactivity elevation and tends to sharply increase dur

Table II Erythrokinetics of neuraminidase-treated rabbit red cells spleen and liver radioactivity before (T/50) and after (T/20) 'hump

Sialic acid release, %	T/50 h	Liver radioactivity[1]		Spleen/liver radioactivity	
		before 'hump	after 'hump	before 'hump	after 'hump
A Total erythrocyte population					
81	11	32.8		1 6	
80	6	26..		2.2	
50	18		14.5		11 0
83	5		16.7		27.8
46	13		8.2		49 0
B. Young erythrocytes					
80	18	30.2		1 6	
82	20	23.5		..2	
78	..3		17.3		8.6
79	24		11 6		7 7
75	"0		16.0		2..3

[1] Liver radioactivity is expressed as percentage of the total injected radioactivity

determined before the rise of blood radioactivity and much higher after this rise (table IIB) This is due to the fact that liver radioactivity is significantly higher before than after hump

Discussion

Sialic acid, as already pointed out by other authors [6 8–10] plays an important role in the determination of the erythrocyte life-span the T/50 value of neuraminidase-treated erythrocytes decreases the higher the percentage of sialic acid released from the membrane. Our results, obtained by using total erythrocyte population, seem to indicate that the T/50 decrease is very sharp when more than 45% sialic acid is removed from the membrane A careful investigation on the [51]Cr survival curves after removal of more than 40–50% sialic acid proved that, after a rapid decrease, blood radioactivity shows an evident increase and reaches a maximum level 50–80 h after injection Radioactivity is totally bound to red

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ing the second slow phase of blood radioactivity decrease This is due to the fact that the liver radioactivity is higher before than after hump It seems probable that liver is not only involved in the destruction of the injured cells but is the site where membrane sialic acid is partially restored in the erythrocytes which will reappear in the circulation At the hepatic level therefore a sialyltransferase activity which restores membrane sialic acid could occur the presence of sialyltransferase on the erythrocyte membrane is excluded by some authors [10] and reported by others [11] mature mammalian erythrocytes cannot in any case, synthesize CMP sialic acid [14] so that it seems more probable that both the enzymes and substrates necessary for sialic acid restoration are extra-erythrocytic

The effects of neuraminidase on a population of young erythrocytes, after treatment of the animals with phenylhydrazine were similar to those produced on a normal erythrocyte population It is relevant that in this case the percentage radioactivity which reappears in the blood is slightly higher than in the experiments in which a normal erythrocyte population was used The restoration of membrane sialic acid could therefore, have a larger extent in young erythrocytes which have not yet undergone the metabolic modifications characteristic of red cell aging Since some evidences [16] suggest that sialic acid content in reticulocyte membrane is lower than in erythrocyte membrane experiments are in progress to investigate if the hepatic homing demonstrated by using neuraminidase treated erythrocytes and phenylhydrazine-induced reticulocytes could play a role in the physiological erythrocyte maturation

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Burkitt's Lymphoma Cell Leukemia

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Key Words Burkitt s lymphoma cell leukemia Leukemia Malignant lymphoma

Abstract To the best of our knowledge only 8 cases of Burkitt s lymphoma cell leukemia were diagnosed whilst the patient was alive. This is a report of one more case and a review of the literature

Burkitt s lymphoma which constitutes more than 50% of all childhood malignant tumors in Africa, was first described by Burkitt [2] in 1958. Outside of Africa, abdominal central nervous system and thyroid involvement are more frequent less frequently jaw bone marrow and peripheral lymph nodes are involved [4 6–9 11 19] However the first case of Burkitt s lymphoma from Turkey was reported in a child with nasopharyngeal tumor [16]

The prevalence of bone marrow infiltration in Burkitt s lymphoma (approximately 8% in African 5–50% in American and 10–33% in Turkish children [5 19]) is less frequent than lymphosarcoma [1 6 14 18 20 22] However Burkitt s lymphoma cell leukemia is much rarer [6–8 12 17 19] and therefore, we would like to report a case of this kind of leukemia.

Case Report

A 5-year-old boy (HCH 671396) was admitted to Hacettepe Children's Hospital with the complaints of paleness, loss of appetite, pain and swelling of the legs, weakness and rapid enlargement of the abdomen of 10 days duration.

[1] We are grateful to Prof M Köksal for his help in the staining of bone marrow slides with methyl green pyronin.



Fig. 1. Burkitt's lymphoma cells in bone marrow aspirate.
Fig. 2. Pyroninophilia in the cytoplasm of Burkitt lymphoma cells.

Physical examination showed an acutely sick looking, pale boy with edema of legs and eyelids. Several lymph nodes in the cervical, axillary and inguinal regions (1×1×1.5 cm) were enlarged. The liver and spleen were 7 and 2 cm palpable below the costal margins, and the collateral veins over the abdomen were prominent. In addition, two masses, one below the liver (8×10 cm) and another (5×5 cm) in the suprapubic region, were palpable. Other physical findings, including neurologic and thyroid examination, were not contributory.

Hb 9.6 g/dl, Hct 29.3%, white cell count 25,299/µl. On the peripheral smear platelets were adequate, and 66% segmented neutrophils, 25% lymphocytes, 9% tumor

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ruricemia and elevated BUN level might also indicate kidney involvement in our patient. With acute leukemia treatment the abdominal masses and lymph nodes regressed simultaneously but, as in other reported Burkitt's lymphoma cell leukemias [6, 7] our patient died within a short period of time (in 25 days)

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*cells with multiple vacuolization were observed. Cellular bone marrow was completely replaced by multiple vacuolated Burkitt's lymphoma cells. These cells had basophilic cytoplasm and fine granula nuclear chromatin with one to five nucleoli [21] (fig. 1). With methyl green pyronin, pyroninophilia of the cells was clearly shown (fig. 2).

An IVP showed enlarged kidneys. Hypoproteinemia (5 g/dl albumin 3.2 g/dl) and hyperuricemia (13.1 mg/dl) were documented. Other laboratory findings, including urinalysis, blood electrolytes, spinal fluid, chest, skull and vertebral X-rays were normal.

With the diagnosis of acute leukemia, the patient was given vincristin (? mg/m²/day orally), methotrexate (12 mg/m²/week, intrathecally) and prednisolone (1?0 mg/m²/day orally). In addition to the above therapy fresh blood transfusions, allopurinol (10 mg/kg, orally) and sodium bicarbonate (100 mg/m²/day) were also given. On the second week of admission the size of the abdominal masses and peripheral lymph nodes regressed and the tumor cells disappeared from the peripheral circulation; the patient was also found to be leukopenic (1,400/µl). On the third week of admission, he was given penicillin (8×300,000 U intravenously) and gentamicin (3 mg/kg/day) because of bronchopneumonia. The infection did not respond to therapy and the patient died 4 days later. Permission for autopsy was not granted; *post mortem* liver micronecropsy did not show any infiltration.

Discussion

Malignant lymphomas, especially lymphosarcoma, transform to leukemia [6] in 15–70% of the cases in children, being somewhat related to the localization and type of cells of the lymphoma [1, 4, 14, 20, 22]. The term *lymphosarcoma cell leukemia* is used for this transformation by some authors [10]. In Burkitt's lymphoma, bone marrow replacement occurs less frequently, but observation of the lymphoma cells in the peripheral blood is rarely seen [6, 15, 22]. BURKITT and O'CONOR [3] stated that they had not seen leukemia in association with Burkitt's lymphoma in Africa.

Imprint preparations of Burkitt's lymphoma cells are identical to those of the cells in the bone marrow if it is infiltrated [7, 22]. In Burkitt's lymphoma, pyroninophilia in the cytoplasm of these cells with methyl green pyronin is fairly characteristic [6, 13], which was shown in the bone marrow cells of this case.

The abdominal lymphoma localization is frequent in Burkitt's lymphoma outside Africa [18, 19] as in this case, but superficial lymph node involvement, which was observed in our patient, is not common in Burkitt's lymphoma [7, 8, 13]. The enlargement of kidneys on the IVP hype

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Effects of Dipyridamole on Sodium and Potassium Content of Human Red Blood Cells[1]

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Key Words. Dipyridamole · Erythrocyte metabolism · Iron deficiency anaemia · Menadione · Red cell cations · Thalassaemia

Abstract. The effects of dipyridamole on potassium and sodium content of human red blood cells from normals or from patients with hypochromic anaemia, and of normal red cells pretreated with menadione, have been studied. Dipyridamole added to the incubation medium decreases the loss of potassium and the accumulation of sodium in all three types of red cells incubated at 37 °C for 24 h, either with or without calcium. The possible mechanism underlying these effects of dipyridamole is discussed.

Dipyridamole is a pyrimido-pyrimidine derivative (2,6-bis[diethanolamino]-4,8-dipiperidino-pyrimido-5,4-d-pyrimidine) currently employed as an inhibitor of platelet aggregation. It has been shown to delay on blood storage the decline *in vitro* in the red cell levels of adenosine triphosphate (ATP) [2, 9], guanosine triphosphate [9] and 2,3-diphosphoglycerate [1, 5, 9]. The effect on ATP might be explained either by an inhibition of adenosine deaminases, as suggested by Gibson and Lionetti [2], or by an inhibition of an unidentified membrane ATPase, as proposed by Duhm *et al.* [1]. Philipp and Banaschak [6] described an inhibition of sodium potassium ATPase by dipyridamole, with a resulting loss of potassium (K^+) and gain of sodium (Na^+) in rabbit and duck red cells incubated over a period of 5 h. Human hypochromic red blood cells (RBC) from patients with β-thalassaemia [3, 7] or iron deficiency anaemia [7] lose

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K and water during metabolic depletion on incubation at 37 °C for 24 h. The K^+ loss can be increased by addition of calcium (Ca^{++}) [8] and prevented by addition of glucose [7] to the incubation medium.

We have previously shown [8] that normal RBC behave like hypochromic cells on incubation *in vitro* when they are pretreated with menadione sodium bisulphite (MSB) The subject of this paper is to describe the effects of dipyridamole on K^+ and Na^+ changes, obtained on incubation *in vitro* of human RBC from normals and individuals with hypochromic anaemia, and of normal RBC pretreated with MSB

Materials and Methods

The experiments were carried out on heparinized blood from 15 normal subjects and from 11 subjects with hypochromic anaemia (6 of them with heterozygous β thalassaemia and 5 with iron deficiency anaemia). Ten normal blood samples were preincubated for 75 min at 37 °C in a buffer solution containing 1 15 mM MSB and 35 mM glucose, as described elsewhere [8], and then washed three times in saline.

The samples from each group were divided in 5 aliquots: one of them was used immediately for the measurement of K and Na^{++} the others were incubated at 37 °C for 24 h in the following fashions: (1) whole blood without added substances; (2) whole blood with the addition of dipyridamole (Persantin® Boehringer Ingelheim, from vials for pharmaceutical use, was directly added to the cell suspension) at the final concentration of 4 mM (concentrations ranged between 3 and 6 mM appeared to be the most active in preliminary experiments) (3) RBC diluted to a haematocrit value between 35 and 45% in a buffer solution, pH 7 4 containing 130 mM NaCl 20 mM tris-HCl, and 1 mM $CaCl_2$, (4) RBC diluted in the buffer solution of the experiment 3 with the addition of dipyridamole at the final concentration of 4 mM.

Untreated and MSB-pretreated cells, whether fresh or incubated, were washed three times with 120 mM $MgCl_2$, and then 0.2 ml of packed cells were lysed in 9.8 ml of 0.1 / Triton-X 100 in distilled water K and Na concentrations were measured on each haemolysate by flame photometry and referred to a constant number of 10^{10} RBC, as calculated from the mean corpuscular haemoglobin content of the original blood and the haemoglobin concentration of each haemolysate.

Results

The mean absolute values of RBC K with standard deviation for normal, MSB-pretreated and hypochromic cells are reported on table I In figure 1 the RBC K changes observed on incubation are expressed as percentage variations as compared with values obtained in the same cells

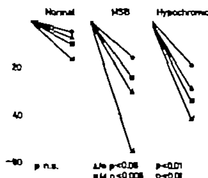


Fig. 1 Mean percentage decreases of K content in normal RBC, in MSB-pretreated cells and in hypochromic cells, incubated at 37°C for 24 h. ▲ = Incubation ● = incubation + dipyridamole ■ = incubation + calcium + dipyridamole ◢ = incubation + calcium.

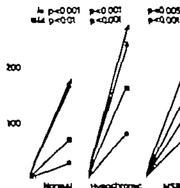


Fig. 2. Mean percentage increases of Na^+ content in normal RBC, in hypochromic cells, and in normal cells pretreated with MSB, after incubation at 37°C for 24 h. For explanation of symbols, see figure 1

before incubation. As can be seen, K loss from red cells incubated without any addition is low in normal cells, but it is much higher in MSB-pretreated cells as well as in hypochromic cells. Addition of Ca^{++} increases slightly the K loss from normal cells, and increases it markedly from hypochromic cells, and even more from MSB-pretreated ones. Dipyridamole partially prevents the K loss, especially from MSB-pretreated and

Table I RBC potassium values (mEq/10[13] cells ±SD)

	Normal n = 15	MSB n = 10	Hypo-chromic n = 11
Fresh RBC	81.9±13.8	84.6±15.4	83.6±11.5
Incubated[1] in plasma	76.4±11.6	62.7±10.0	60.4±15.0
Incubation[1]+dipyridamole	77.8±10.6	75.7±9.7	67.6±13.5
Incubation[1]+calcium	66.4±9.9	44.6±25.3	48.5±20.5
Incubation[1]+dipyridamole+calcium	67.8±9.4	65.7±14.1	52.5±17.4

[1] Incubation at 37 C for 24 h.

Table II RBC sodium values (mEq/10[13] cells ±SD)

	Normal n = 15	MSB n = 10	Hypo-chromic n = 11
Fresh RBC	5.25±1.77	6.43±2.11	4.34±1.88
Incubated[1] in plasma	13.39±3.01	13.94±1.18	12.44±2.90
Incubation[1]+dipyridamole	6.03±1.29	8.43±2.38	6.52±1.53
Incubation[1]+calcium	12.74±1.85	18.18±6.67	15.72±5.21
Incubation[1]+dipyridamole+calcium	8.10±1.57	12.54±4.18	11.00±4.34

[1] Incubation at 37°C for 24 h.

from hypochromic cells. The effect of dipyridamole is also evident in presence of Ca^{++}

Table II reports the mean absolute values of Na in normal, MSB-pretreated and hypochromic cells. In figure 2 the percentage variations of Na obtained on incubation of red cells of the same three groups, are shown. The increase in red cell Na observed on incubation without any addition is almost the same in normal and in MSB-pretreated cells, but it is appreciably higher in hypochromic cells. In presence of Ca^{++} the increase of Na was greater particularly in the hypochromic and MSB-pretreated cells. Dipyridamole largely prevents the increase of Na in all



Fig. 3. RBC Na values at different times of incubation at 37 °C, either with (■) or without (●) dipyridamole.

three types of incubated cells. It is also effective in reducing the accumulation of Na in presence of Ca^{++}

Figure 3 depicts the RBC Na values at different times during 24-hour incubation either in absence or in presence of dipyridamole. It confirms that dipyridamole delays and reduces the gain of Na

Discussion

The effects of dipyridamole on cation content of incubated RBC can be summarized as follows: (1) Dipyridamole reduces the K loss and Na gain, which are observed when the red cells are incubated in absence of exogenous substrates and become depleted of glucose. (2) This effect of dipyridamole also occurs in the presence of Ca^{++}

The exact mechanism underlying these effects of dipyridamole on cation content of RBC is not known. Our present findings are not in agreement with those of PHILIPP and BAMABCHAK [6] whether the discrepancy between our results and theirs is due to the different experimental conditions or to species differences remains to be established.

Since in human RBC dipyridamole appears to improve the cation transport, we must assume that, if an ATPase is inhibited by the drug, this should be an ATPase other than the enzyme concerned with the operation of the Na K pump. Our data suggest that the pump enzyme operates more efficiently in the cells incubated with dipyridamole, possibly because of a greater availability of ATP which may be a consequence of the

ATP-sparing effect of the drug. This effect seems supposable also in the absence of added adenosine (as in our experiments) but in this case it requires concentrations of dipyridamole about ten times higher than those used by GIBSON and LIONETTI [2] and by ZACHARA [9]

Finally it is well known that Ca^{++} enhances the K^+ leakage from red cells when ATP decreases [4] This effect is particularly evident in hypochromic and in normal MSB-pretreated cells. Probably dipyridamole is partially able to prevent the loss of K from RBC incubated in presence of Ca^{++} through an adequate availability of ATP which delays the leaking effect of Ca^{++}

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Peripheral Blood Lymphocytes in Systemic Lupus erythematosus

Relation to Activity

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Key Words. Drug-induced lupus E-rosette-forming cells Immunoglobulin-bearing cells Lupus erythematosus Lymphocytes

Abstract. Peripheral blood lymphocytes from 20 patients with systemic lupus erythematosus (SLE), 3 patients with drug-induced lupus and 20 normal controls were studied. The absolute number of E-rosette-forming cells (ERFC) and surface immunoglobulin-bearing cells were determined during active and inactive stages of the disease. An attempt was made to establish the relationship between the number of ERFC and the clinical stages of the disease on one hand and treatment on the other. A decrease in ERFC was observed in all SLE patients, but it was most prominent in patients with active disease. No correlation was found between treatment and the decreased numbers of ERFC.

Considerable evidence has accumulated for the existence of immunological alterations, both humoral and cellular in systemic lupus erythematosus (SLE) [1 3 5 10] A decrease in peripheral thymus-derived lymphocytes (T cells) in activ SLE has been described [15 17] However the question remains open whether the disease *per se* or the treatment is responsible for it [26]

In the present study E rosette-forming cells (ERFC) and surface immunoglobulin-bearing (SIg) cells were determined in 20 patients with SLE and 3 patients with drug-induced lupus (DIL), the first being a well-known marker for thymus-derived lymphocytes and the second for bone-marrow-derived lymphocytes (B cells). Emphasis was laid on the correlation between the disease activity therapy and the peripheral distribution of the above-mentioned cells.

Part of this work was submitted to the Sackler School of Medicine, Tel-Aviv University in partial fulfillment of the requirements for the degree of MD

Clinical Material and Methods

23 patients with SLE and DIL (most of them followed in the Hematology Department of the Chaim Sheba Medical Center) and 20 normal controls, matched for age and sex, were included in this study The diagnosis was made according to the criteria established by the American Rheumatism Association [6] Three groups of patients were studied.

(1) Eight patients with active disease, as manifested by the presence of fever arthritis, skin rash, serositis, progressive renal damage, thrombocytopenia and hemolytic anemia, frequently accompanied by a decrease in serum complement levels. Three of them were studied prior to any therapy and were restudied after clinical improvement.

(2) Twelve patients without signs of active disease, eight of them on maintenance therapy The treated patients were receiving steroids alone or in combination with imuran or cyclophosphamide (table II).

(3) Three patients with DIL, one after practolol therapy the second after procaine amide and the third after mesantoin. Two of them had signs of active disease at the time of testing. These patients were neither on immunosuppressive therapy nor on steroids.

Laboratory studies. Peripheral blood was obtained in heparin and the mononuclear cells separated on a Ficoll metrizoate gradient, as previously described [4]. The preparations contained at least 90% lymphocytes. E rosettes were determined by a slight modification of the method of Wybran [28] briefly 0.25 ml of lymphocyte suspension containing 1×10^6 cells was mixed with 0.25 ml of a 1% suspension of fresh sheep red cells and 50 μl of calf serum. After a ?-min centrifugation at 200 *g* the mixture was incubated at 18 °C for 1 h. After gentle mixing the ERFC were counted, by means of a phase microscope (Zeiss). SIg cells were enumerated, using direct immunofluorescence according to the method described by Taylor *et al.* [21] The monocytes were excluded morphologically by the phase-contrast microscope. This was possible after finding a good correlation between the phase-contrast microscopy on one hand and α-naphtyl-acetate esterase and peroxidase staining as well as EA rosettes, according to Shevach *et al.* [19], on the other Absolute lymphocyte counts were calculated from total and differential leukocyte counts. Student's t test was used for the statistical analysis.

Results

E-rosette-forming cells As seen in table I the number of ERFC in the SLE patients was significantly lower than in the normal controls ($p < 0.0005$) This observation is evident when expressed both in percent and absolute number of cells. Furthermore, a decrease in ERFC was most striking in active disease independent of therapy (table II) The mean percent of ERFC in acutely ill patients was 29 6 and the total number/mm³ was 303 8 These were significantly lower than the 50% and

Table I. ERFC and surface SIg cells in SLE patients and normal controls (mean ± SD)

	ERFC		SIg	
	%	per mm³	%	per mm³
SLE (= 20)	41.8 ± 13.5	496.15 ± 300.92	37.9 ± 12.4	461.3 ± 318.62
Normals (n = 20)	63.5 ± 4.32	1,134 ± 125.6	34.5 ± 3.86	434.10 ± 64.65
		$p < 0.0005$		not significant

Table II ERFC and SIg cells in active and inactive SLE patients

Therapy mg/day	Lymphocytes/ mm³	ERFC %	per mm³	SIg %	per mm³
Active					
Pr,30	441	30	132	38	168
No	833	33	274	36	300
Pr,60 Im,100	924	40	380	50	465
No	1,608	22	354	61	981
Pr,15	880	20	176	32	282
Pr,20	1,200	50	600	40	480
No	1,600	20	320	52	832
No	890	22	196	59	525
Mean ± SD	1,047 ± 400.8	29.62 ± 10.92	303.8 ± 148.6	46 ± 10.96	504 ± 278
Inactive					
Pr 40	742	48	356	38	282
Pr,20	2,592	40	1,036	53	1,372
No	858	50	429	18	157
Pr 7.5	2,030	48	974	31	629
No	1,800	60	1,080	39	602
Pr,5 Im,50	626	42	262	22	138
Pr,30	1,590	48	763	40	636
No	1,156	48	555	37	428
No	440	50	220	42	184
Pr,15 Im,100	780	42	328	18	140
Pr,5	1,200	52	624	32	384
Pr,30	1,200	72	864	20	240
Mean ± SD	1,230.91 ± 640.68	50 ± 8.69	624.25 ± 312.4	32.5 ± 11.0	432.7 ± 351.9

Active versus inactive lymphocytes/mm = not significant ERFC/mm = $p < 0.01$ SIg/mm = not significant. Pr = Prednisone Im = Imuran.

Table III ERFC and SIg cells before treatment and after clinical improvement in patients with active disease

Patients	Before treatment			After 2 months		
	lymphocytes/mm³	ERFC %	SIg, %	lymphocytes/mm³	ERFC %	SIg, %
P D[1]	1 608	22	61	1 770	48	18
S. O.[1]	1 600	20	52	1 680	42	23
K. O	890	22	59		still active	

[1] P D and S. O. were on 60 mg prednisone/day at the time of the second examination, without signs of activity

Table IV ERFC in active SLE and DIL patients (mean ± SD)

	Lymphocytes/mm³	ERFC %	ERFC per mm³
SLE (n = 8)	1 047 ± 400.8	29 6 ± 10.9	303.8 ± 148.6
DIL (n = 3)	1 446.7 ± 400.7	61 ± 2.64	876 ± 201.6
			p < 0.0005

624.2/mm³ respectively recorded in the patients with inactive disease. The data on each individual patient of these two groups are presented in table II. Three patients with active disease tested before treatment had the lowest numbers of ERFC, although none of them was found to be lymphopenic. In two of them the ERFC rose after clinical improvement and this under high doses of steroids (table III) The DIL patients with active disease had a significantly higher number of ERFC than active SLE patients (p 0 0005 table IV) No correlation was found between the total number of lymphocytes and their ability to form rosettes with sheep erythrocytes (table II).

Surface immunoglobulin-bearing cells No significant differences were found in the absolute number of SIg cells between controls and the patients. A comparison between active and inactive disease revealed no significant differences in the total number/mm³ of SIg cells. However the mean percent of these cells was higher in the acutely ill patients than in those with well-controlled disease (46 versus 32.5 table II) In three patients with signs of clinical activity the increase in SIg cells was more evident prior to any therapy Their number declined following clinical im

provement, simultaneously with the increase in the number of ERFC (table III).

Discussion

Our data clearly indicate that there is a decrease in the absolute numbers of peripheral ERFC in patients with SLE, especially in the active stage of the disease. Moreover there was an increase in the number of ERFC with clinical improvement. By repeated testing of newly diagnosed patients, prior to any treatment, and then while on high doses of corticosteroids and/or on cytotoxic agents, it was shown that the decrease in the number of ERFC was not directly related to the therapeutic agents but rather to the change in the clinical status (table III).

It was surprising to note that in the small group of DIL patients, even with clinical signs of activity no depression in the number of ERFC was observed. Whether this test could be of clinical value in the differential diagnosis between DIL and SLE patients remains to be determined on a larger series of cases.

It has recently become increasingly apparent that there is a close relationship between active SLE and altered cellular immunity as measured by several parameters [7 15 16] A depression in peripheral-blood T cells has been recorded both in humans and animals with autoimmune disease [17 20] Whether this reduction is due to the presence of lymphotoxic antibodies which have been demonstrated in SLE is yet unclear [11, 12, 14 22, 24 25].

Studies have shown a depression of cell-mediated immunity during viral infection [8, 13] Viruses have also been postulated as one of the causes of SLE [18] This agent could directly affect T-cell function resulting in a depression of cellular immunity or indirectly a depression in suppressor T-cell activity [2] inducing the B cells to produce antibodies against T cells.

There are controversial reports concerning the number of SIg cells in SLE [15 23 26, 27] It appeared from our studies that patients with active disease tended to have a higher percent of such cells. However a statistical analysis of the whole group of patients as compared to normals revealed no significant differences in the total number/mm³ of SIg cells. We therefore think that the distribution of B and T cells in the peripheral blood should be expressed in absolute numbers and not in percent. The elevated number of SIg cells found by some authors in SLE [9 27] could

be interpreted along several lines. It may be due to nonspecific adsorption of immunoglobulins on the surface of different lymphocytes or due to specific antilymphocyte antibodies [12, 14 22]

The question remains open whether the decrease in ERFC in SLE is due to a depletion of T cells, adherence of antilymphocyte antibodies on the lymphocytes surface, or to other causes which bring about alterations in the cell surface markers. Further studies are needed in order to elucidate some of the questions raised.

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Cold Agglutinins in a Case of Chronic Lymphatic Leukaemia

A Study of the Lymphocyte Surface

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Key Words. Cold agglutinins · Immunoglobulins · Lymphatic leukaemia · Lymphocyte surface markers

Abstract. A case of chronic lymphatic leukaemia with cold agglutinins is presented in which peripheral blood lymphocytes of varying maturity appeared to possess T- and B-cell markers. A cold agglutinin IgM in type is identified in the serum and at the cell surface. This seems to be responsible for the spontaneous rosetting of sheep red blood cells (SRBC). A single clonal origin of these cells is suggested with maturation arrest. It is also suggested that with configurational change in lymphocyte surface cryoglobulins may sometimes produce spontaneous rosetting with SRBC.

In recent years the use of cytochemical and immunofluorescence techniques has provided more precise identification of the various expressions of leukaemia, particularly those of the lymphoid cell lines [1–4]. We report here a case of lymphatic leukaemia in which a cold agglutinin was identified at the cellular level.

Case History

This patient is a 64-year-old white male who presented with anaemia and fatigue. On physical examination his spleen was enlarged and small lymph nodes were palpable in both inguinal regions. His liver was not enlarged. Laboratory examinations revealed a haemoglobulin of 4.6 g%, the erythrocytes were normocytic and normochromic. The total leucocyte count was 93,000/µl, 93% of these were lymphocytes, 25% of which had an indented nucleus with a prominent nucleolus and a prominent cytoplasm. The majority of the lymphocytes were small and typical of chronic lymphatic leukaemia. Monocytes were not seen. The platelets were 104,000/

μl and the erythrocyte sedimentation rate 105 mm (Westergren). Cold agglutinins were present in the serum in a titre of 1:2,000. Serum bilirubin was 1.9 mg. Total serum protein was normal. Immunoelectrophoresis identified an abnormal IgM K-type; a cryoglobulin was also present. In the bone marrow 35% of the lymphoid cells were periodic acid-Schiff (PAS)-positive and acid-phosphatase-negative. The PAS-positive material was finely granular with a perinuclear distribution.

Materials and Methods

Lymphocyte marker studies. 20–40 ml of heparinized blood were collected and the erythrocytes sedimented using plasmagel (Laboratoire Roger Bellon). The leucocyte-rich suspension was then layered onto Ficoll/Triosil (Pharmacia Fine Chemicals) gradient, SG 1.076. After centrifugation at 260 *g* the lymphocyte layer was removed from the interface, washed three times with cold phosphate-buffered saline (PBS) and the cell count adjusted to 1×10^7/ml for fluorescence studies.

Immunofluorescence studies. Aliquots containing 1×10^6 lymphocytes were spun at 260 *g* and the supernatant discarded. 0.1-ml aliquots of suitably diluted fluorescein-conjugated goat anti-human IgG IgA, IgM, anti-complement (C3) (Baxter Hyland) and rhodamine-conjugated sheep anti-human Fab (Medlabs) were added to the tubes, mixed and kept on ice for 30 min. The cells were washed further three times in cold PBS, the lymphocyte button was then resuspended in one drop of 0.1 M glycine/glycerol buffer pH 8.6, and examined using Zeiss Universal Microscope with ploem illumination system and incident fluorescent light.

A similar procedure was used to determine whether cold agglutinins from patient's serum would react with normal donor lymphocytes and whether or not these donor lymphocytes would then form rosettes with 0+ cells. Lymphocyte suspensions were incubated at 4 °C for 30 min in 0.1-ml volumes of patient's serum diluted 1:5. After washing three times in cold PBS the cells were treated with the above conjugates and O+ erythrocytes.

Rosetting techniques. Fresh SRBC and human O+ erythrocytes were washed three times in PBS and 1% v/v suspensions made up. 0.5 ml of 3×10^6 lymphocytes was added to each of the 0.5-ml volumes of 1% SRBC and 1% 0+ cells. The suspensions were mixed, placed in a water bath for 5 min, spun at 260 *g* and then left at 4 °C for 1 h. *One drop of 0.01% acridine orange was added to the tubes, the cells* were then gently resuspended. One drop was placed on cold glass slide and examined immediately using phase-contrast bright light with incident fluorescent light. A minimum of three adherent red cells per lymphocyte was the criterion for a rosette.

Immunofluorescence staining of patient' rosettes. Using human O+ cells as described above, rosettes were formed After the 1-hour period at 4 °C the supernatant was discarded and 0 1 ml of anti-human *IgM* was added to the tubes, the cells were gently resuspended and incubated at 4 °C for further *30 min* and washed gently three times with cold BPS. Acridine orange was excluded and all rosetting cells were examined for surface IgM using specific antisera.

Inhibition of rosetting. Lymphocytes, 1×10^7/ml, were spun to button and incubated with 0.1 ml of rhodamine-conjugated sheep anti-human Fab for 30 min at

4 °C. The cells were then washed three times with PBS and adjusted to 3×10⁶/ml. The rosetting procedure described above was followed using O+ cells.

Detection of cold agglutinins. The patient's serum was diluted in PBS from 1:2 to 1:4096. Equal volumes of 2% O+ cells and SRBC were added. After overnight incubation at 4 °C the agglutination titres were read. The same technique was used to detect warm agglutinins to O+ and SRBC with the exception that the tubes were incubated at 37 °C overnight.

Results

The results of these studies are summarized as follows.

Rosetting tests. (1) T-cell rosettes: 75%. (2) Rosettes using human O+ erythrocytes: 95%. (3) Rosettes using unsensitized SRBC following 30-min and 12-hour incubation at 37 °C: 50%. (4) Rosettes after pre-incubation of patient's lymphocytes with sheep anti human Fab: 12%.

Immunofluorescence tests. (5) Using anti IgG: 0%; using anti-IgA: 0%; using anti IgM: 100%; using anti-C3: 0%; using anti Fab: 100%; using anti K: 100%; using anti-λ: 0%. (6) Phase and fluorescence microscopy showed that all SRBC rosetting cells carried surface immunoglobulin. (7) Normal donor lymphocytes + patient's serum + fluorescein-conjugated anti human IgM: 100% (control 8%). (8) Normal donor lymphocytes + patient's serum + O-erythrocytes: 0% rosettes. (9) Cold agglutinin titre in patient's serum using SRBC: 1:256; cold agglutinin titre in patient's serum using human O+ erythrocytes: 1:2,000; agglutinin titre at 37 °C using SRBC: 1:8; agglutinin titre at 37 °C using O+ erythrocytes: 0. (10) Lymphocyte rosettes using cord blood erythrocytes from several donors: 70%.

Discussion

It was the initial observations that large numbers of leukaemic lymphocytes from this patient's peripheral blood formed rosettes with unsensitized sheep erythrocytes as well as showing 100% fluorescence using anti-human IgM which prompted further work-up in this case.

The nature of the bond between the erythrocytes and the surface of the lymphocyte was pursued in steps 2, 3, 4, 6 and 8. Human O+ erythrocytes do not normally form spontaneous rosettes with human lymphocytes. Therefore, this phenomenon is directly related to a surface property of the lymphocytes in this case. It does not appear to be due to absorption

of a cold agglutinin from the serum since normal donor lymphocytes, following incubation with the patient's serum, failed to bind erythrocytes (step 8) even though the lymphocytes could in fact absorb IgM from the patient's serum (step 7). Step 6 indicated that all the patient's lymphocytes which formed SRBC rosettes carried surface IgM. The configuration of these rosetting receptor sites vis-à vis the immunoglobulin receptors were revealed in step 4 which indicated that Fab fragment plays an important part in the spontaneous rosetting phenomenon in this case. Step 9 characterizes the reaction of patient's serum with human and sheep erythrocytes. The results here indicate that erythrocyte binding is greater at 4 C for human O+ erythrocytes than for SRBC. At 37 C there is no agglutination of human erythrocytes and a marked drop in titre of SRBC agglutinins. The results of step 3 indicate that the binding of erythrocytes by a cold agglutinin is more stable at the cellular level. Our conclusions are that IgM possessing cold agglutinin activity is present at the cell surface in this case and that spontaneous rosetting is directly related to the Fab portion of the molecule. This agglutinin is present in the serum. The lymphocytes in the peripheral blood appear to be a major source of the agglutinins since few plasma cells were seen in the bone marrow. Further maturation of these lymphocytes might result in Waldenström's macroglobulinaemia with its associated bone marrow findings. The properties of cold agglutinins in cases such as this have been described, and antibody activity directed towards the blood group specificities i, I, and Pr have been identified [5]. The most frequently occurring cold agglutinins are Anti-I [6]. The I antigen is not expressed in cord erythrocytes, so it is reasonable to assume that the rosettes observed in step 10 are due to the presence of receptors for antigens other than I.

Apart from the above observations, these findings indicate potential pitfalls in the interpretation of spontaneous rosettes. These have been alluded to recently [7, 8]. Three possibilities to account for the double marker on lymphocytes in some patients with lymphatic leukaemia have been suggested (1) absorption of cytophilic antibody by T cells, (2) the existence of a subpopulation of B cells which can form SRBC rosettes and (3) the existence of a population of lymphocytes which may function as either T or B cells.

The patient was treated with chlorambucil and his lymphocyte count and other haematological parameters returned to near normal levels. However the monoclonal nature of the circulating lymphocytes did not change significantly. More than 70% of the lymphocytes still bore IgM,

4 °C. The cells were then washed three times with PBS and adjusted to 3×10⁶/ml. The rosetting procedure described above was followed using O+ cells.

Detection of cold agglutinins The patient s serum was diluted in PBS from 1.2 to 1 4096. Equal volumes of 2% O+ cells and SRBC were added. After overnight incubation at 4 C the agglutination titres were read. The same technique was used to detect warm agglutinins to O+ and SRBC with the exception that the tubes were incubated at 37 °C overnight.

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Rosetting tests (1) T-cell rosettes 75%. (2) Rosettes using human O+ erythrocytes 95%. (3) Rosettes using unsensitized SRBC following 30-min and 12 hour incubation at 37 C. 50%. (4) Rosettes after pre-incubation of patient s lymphocytes with sheep anti-human Fab 12%

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It was the initial observations that large numbers of leukaemic lymphocytes from this patient s peripheral blood formed rosettes with unsensitized sheep erythrocytes as well as showing 100% fluorescence using anti-human IgM which prompted further work-up in this case.

The nature of the bond between the erythrocytes and the surface of the lymphocyte was pursued in steps 2, 3 4 6 and 8 Human O+ erythrocytes do not normally form spontaneous rosettes with human lymphocytes. Therefore this phenomenon is directly related to a surface property of the lymphocytes in this case. It does not appear to be due to absorption

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Peptichemio: A New Oncolytic Drug in Combination with Vincristine and Prednisolone in the Treatment of Non-Hodgkin Lymphomas

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Key Words. Chemotherapy · Malignant lymphomas · Non-Hodgkin lymphomas · Peptichemio · PVP

Abstract. Peptichemio, a new oncolytic drug with alkylating and antimetabolic properties was employed in combination with vincristine and 6-methylprednisolone (PVP) for the treatment of diffuse non-Hodgkin's lymphomas (NHL), stages III and IV. 52 of 66 patients who entered the PVP protocol were considered evaluable: the overall remission rate in diffuse lymphocytic poorly differentiated lymphomas (DLPD) was 56.6 and 55% in diffuse histiocytic lymphomas (DHL). Median duration of complete remission was 16 months in DLPD and 13 months in DHL. Dramatic results were obtained in 'oncologic emergencies' such as mediastinal involvement and spinal cord compression. Bone marrow depression from PVP was moderate and never caused discontinuation of therapy. According to these results the PVP protocol would appear susceptible of offering a major contribution to the chemotherapy of diffuse NHL.

Peptichemio (PTC) is a multipeptide complex synthesized by DE BARBIERI *et al.* [7–9]. It consists of six peptides conjugated at the amino and the carboxyl group of the *m*-[di-(2-chloroethyl)-amino]-L-phenyl alanine molecule by means of covalent bonds (fig. 1). Its alkylating activity is due to the dichlorodiethylamino $[-N-(CH_2CH_2Cl)]_2$ group in the m-position of phenylalanine [13, 14], while the six peptides bound in the same molecule would be responsible for the antimetabolic effect of the compound.

Clinical trials were started by our group since 1970 and the results were reported extensively elsewhere [6, 26, 27]. After having established the drug's remarkable and versatile oncolytic properties in a wide spec

K-type on their surface This observation poses important questions concerning the nature of the disease in this condition.

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Table I. Distribution of patients according to stage and histologic type

Stage	Histologic type					
	DLPD		ML		DHL	
	A	B	A	B	A	B
II						2
III	2	19			2	6
III E		3				
IV	2	4		2	2	8
Total	30				20	

Table II. PVP protocol: Peptichemio, vincristine and 6-methylprednisolone

Drugs	Dosage, mg/m² ( )	
	with bone marrow involvement	without bone marrow involvement
Peptichemio	25 (day 1, 2, [3])	50 (day 1, 2, 3)
Vincristine	1 (day 1)	1.4 (day 1)
6-Methylprednisolone	40 (days 1–7)	40 (days 1–7)

great majority was shown to consist of B-cell lymphomas and immunoblastic sarcomas; however, these results will be reported elsewhere and the term of histiocytic lymphomas will be still adhered to in this contribution [29].

Clinical features. The clinical stage according to Ann Arbor' criteria [5] was established on the basis of physical examination, lymphangiography, usual routine laboratory investigations and X-ray surveys, bone marrow needle biopsies were performed in all cases. Staging laparotomies were not performed because of the advanced stage of the disease in patients who entered the present study. Table I includes 11 cases classified according to the clinical stage and to the histologic type.

Diffuse lymphocytic poorly differentiated lymphomas (DLPD). 30 patients ere classified as DLPD, 67% of which were males, mean age being 47 years; of these 10% were admitted with pleural effusion and cytocentrifuge investigations confirmed the presence of lymphoma cells in the sediment. One case underwent leukaemic transformation followed by death after 14 months of complete remission maintained with PVP.

Mixed lymphocytic lymphoma (ML) was the diagnosis of tw patients, both males.

Diffuse histiocytic lymphomas (DHL). The remaining 20 cases were classified as DHL, 75% of which males, with mean age of 52 years. Of these 10% were

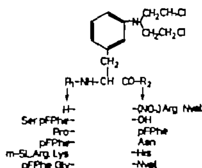


Fig 1 Peptichemio chemical formula.

trum of neoplastic diseases, attention was focused on haematologic ma lignancies. Thus, at present, our experience includes more than 600 cases of blood diseases and solid neoplasms treated with PTC either alone or in combination with other drugs The most encouraging results were obtained mainly in non Hodgkin lymphomas (NHL) as well as in multiple myeloma however PTC's efficacy in chronic lymphocytic and chronic myelogenous leukaemia in Waldenström s macroglobulinaemia, and in myelofibrosis with myeloid metaplasia of the spleen was also re- markable. The role of PTC in the management of acute leukaemias can not yet be established on the basis of our experience, whereas some pos itive results were observed in solid neoplasms. Among the latter special emphasis should be given to the association with 5 Fluorouracil for the treatment of gastrointestinal carcinomas, with methotrexate in lung car cinomas, and with adriamycin for disseminated malignancies of un known origin

The present report concerns the association of PTC with vincristine (VCR) and 6-methylprednisolone for the treatment of advanced NHL (PVP protocol) The abbreviation PVP was maintained even when intra venous dexamethasone instead of 6-methylprednisolone was employed

Patients and Methods

The present study includes 52 previously untreated cases of NHL.

Histo-cytological investigations RAPPAPORT's [31] classification of NHL was employed for histologic diagnosis of original biopsy specimens 11 cases of so-called histiocytic lymphomas were investigated with newer immunologic methods (AHLG immunofluorescence test [28], methods for T and B lymphocytes), and the

Table I Distribution of patients according to stage and histologic type

Stage	Histologic type					
	DLPD		ML		DHL	
	A	B	A	B	A	B
II						2
III	2	19			2	6
III E		3				
IV		4		2	2	8
Total	30		2		20	

Table II PVP protocol Peptichemio, vincristine and 6-methylprednisolone

Drugs	Dosage, mg/m ( ) with bone marrow involvement	without bone marrow involvement
Peptichemio	25 (day 1 2, [3])	50 (day 1 2, 3)
Vincristine	1 (day 1)	1 4 (day 1)
6-Methylprednisolone	40 (days 1 7)	40 (days 1 7)

great majority was shown to consist of B-cell lymphomas and immunoblastic sarcomas; however these results will be reported elsewhere and the term of histiocytic lymphomas will be still adhered to in this contribution [29].

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Diffuse lymphocytic poorly differentiated lymphomas (DLPD). 30 patients were classified as DLPD, 67° of which were males, mean age being 47 years; of these 10° were admitted with a pleural effusion and cytocentrifuge investigations confirmed the presence of lymphoma cells in the sediment. One case underwent leukaemic transformation followed by death fter 14 months of complete remission maintained th PVP

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Table III PVP in NHL relapsed after treatment with combinations including CTX

Histology	Number of cases	Response CR	PR	NR	Duration of CR months	Alive	Dead
DLPD	10	2	4	4	3	4	6
DHL	4	2	1	1	3	2	
Total	14	4	5	5	3	6	8
		9 (64%)					

admitted with a pleural effusion, 5% with a primitive localization of Waldeyer's ring and 5% with a primitive gastrointestinal localization. In one patient DHL developed *after a 4-year history of chronic lymphocytic leukaemia, thus exhibiting* the features of the so-called Richter syndrome [23, 32]

Drugs and dosage Dosage schedules of the PVP protocol are reported in table II. Two different schedules were followed, according to the results of the bone marrow biopsies: in patients with bone marrow involvement [37] (33% of all cases) the dosage of PTC and VCR were reduced of 40%. Each ampoule of PTC containing 40 mg of active compound, was diluted with 125 ml of a 5% glucose solution and administered by intravenous infusion within 1 h. Every course of treatment lasted 3 days and was repeated every 3 weeks. In the interval androgens were administered at the dosage of 100 mg of testosterone-enanthate weekly Courses after the first were given on an outpatient basis. When PTC was employed for local therapy in *neoplastic serositis*, it was diluted in 20 ml of a 5% glucose solution and introduced in the pleural cavity after removal of the fluid 40 mg of PTC were employed for each administration. Average schedules for *mediastinal syndrome* or *spinal cord compressions* were PTC 200 mg (100 mg/day) plus VCR 1.4 mg/m (or 1 mg, according to the presence of bone marrow involvement) on day 1 and 6-methylprednisolone 40 mg/m /day by intravenous infusion for 7 days, a single dose of 200 mg of PTC on day 1 was employed in a case of extreme mediastinal involvement (table V)

Response The criteria for the evaluation of responses to the PVP therapy were the following:
Complete remission (CR) complete regression of all physical signs, as well as complete disappearance of subjective symptoms of the disease Partial remission (PR) improvement greater than 50% of physical signs, with marked reduction of subjective symptoms. Refractoriness (NR) no improvement whatsoever

Results

The total number of patients admitted to the PVP protocol includes 66 cases, of which only 52 are being considered in the present study

Table IV Remission rates and duration by histology

Histology	Number of cases	Evaluable	Remission rates CR	PR	NR	Duration of CR months
DLPD	40	30	17 (56.6%)	8 (26.5%)	5 (16.6%)	16
ML	2	2	2			10
DHL	24	20	11 (55%)	6 (30%)	3 (15%)	13
Total	66	52	30	14	8	
			44			

Table V Results obtained with PVP in 'oncologic emergencies'

Case No.	Histology	Stage	Localization	Results	Dosage of PTC mg
1	DLPD	III B	mediastinum	CR	200
2	DLPD	III B	mediastinum	CR	200
3	DLPD	IV A	mediastinum	CR	200
4	DHL	IV B	epidural	CR	160
5	DHL	III B	epidural	CR	160
6	DLPD	III B	epidural	CR	200
7	DHL	III B	epidural	NR	160

The 14 patients considered non-evaluable had been previously treated according to other protocols, and were subsequently given PVP after proven refractoriness to other combinations. Of this group of patients 9 (64%) obtained either a CR (4) or a PR (5) as outlined in table III their median remission duration was 3 months, but 6 patients are still alive and in PVP therapy Of the same group 8 patients died of complications related to the disease

The percentage of CR among the previously untreated patients was 56% in DLPD and 55% in DHL (table IV) If one adds CR rates to PR rate, the overall remission rate with the PVP protocol is 84.5%, which may be regarded as among the highest response figures when compared to other combinations. The median remission duration was 16 months for the DLPD and 13 months for the DHL group 40% of the patients are still alive, but only 20% of them are in complete remission. Survival

Table III PVP in NHL relapsed after treatment with combinations including CTX

Histology	Number of cases	Response CR	PR	NR	Duration of CR months	Alive	Dead
DLPD	10	2	4	4	3	4	6
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1	DLPD	III B	mediastinum	CR	200
2	DLPD	III B	mediastinum	CR	200
3	DLPD	IV A	mediastinum	CR	200
4	DHL	IV B	epidural	CR	160
5	DHL	III B	epidural	CR	160
6	DLPD	III B	epidural	CR	200
7	DHL	III B	epidural	NR	160

The 14 patients considered non-evaluable had been previously treated according to other protocols, and were subsequently given PVP after proven refractoriness to other combinations. Of this group of patients 9 (64%) obtained either a CR (4) or a PR (5) as outlined in table III their median remission duration was 3 months, but 6 patients are still alive and in PVP therapy Of the same group 8 patients died of complications related to the disease.

The percentage of CR among the previously untreated patients was 56% in DLPD and 55 % in DHL (table IV). If one adds CR rates to PR rate, the overall remission rate with the PVP protocol is 84.5 %, which may be regarded as among the highest response figures when compared to other combinations. The median remission duration was 16 months for the DLPD and 13 months for the DHL group 40% of the patients are still alive, but only 20% of them are in complete remission. Survival



Fig 2 Neoplastic serositis in a case of DLPD X ray examination before (a) and after (b) intrapleural administration of 120 mg PTC.

was determined starting from the 1st day of PVP treatment and varied from 12 to 22 months for the DLPD and from 8 to 17 months for the DHL. It is probable that survival curves will still increase, since 25% of the patients are in CR and 50% are still alive

Neoplastic serositis was found in 10% of the patients, 80% of which responded to PTC administered locally 2–3 evacuations were generally

Table VI. Toxicity phenomena from PVP therapy

Symptoms	Cases %
Vein thrombosis	89
Alopecia	80
Anorexia	80
Myelosuppression	52
Nausea	40
Peripheral neuropathy	35

necessary before total disappearance of pleural effusion (fig. 2), with a total dosage of PTC never less than 120 mg. The tolerance was good, but some patients complained of vague thoracic pains for 24 h after the administrations of the drug.

Mediastinal syndromes (table V) were treated with high-dosage schedules of PVP: in three patients, complete disapperance of compressive phenomena was evident within 72 h after the administration of the drug by intravenous infusion, the first signs of general improvement appearing already a few hours after PVP had been administered. X-ray examinations 1 week after the first course of PVP usually showed considerable improvement (fig. 3, 4). All these three patients are living at present.

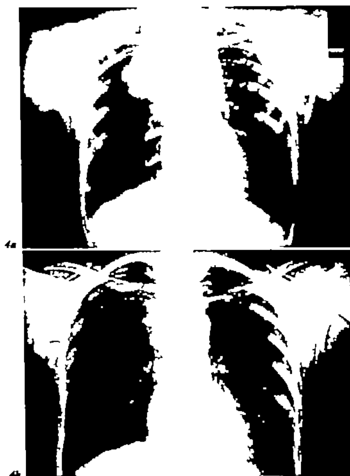
Four paraplegic patients with epidural compression were similarly treated with PVP: in three cases ambulation was generally restored after 8 days from the first PVP course, with the first signs of amelioration sometimes occurring already a few hours after the infusion of the drug. These three patients are all c and in remission (16, 13 and 11 months, respectively), whereas a fourth patient showed no signs of improvement.

Toxicity. Toxicity phenomena, secondary to PTC administrations, are outlined in table VI. Phlebothrombosis represented the most serious complication and occurred in almost 80% of cases; however, subsequent addition of pyridinolcarbamate, as suggested by DE BARBIERI *et al.* [8] and regularly performed since 1974, reduced the occurrence of thrombosis of the vein at the site of injection very significantly. Alopecia was quite common, but hair started growing again normally 2 weeks after the discontinuation of therapy. Nausea and vomiting were considered minor side-effects and, if present, they could be corrected by the administration of sulpyride and chlorpromazine. Although PTC has a potent myelosup-



Fig 3 4 Serial chest radiographs, showing mediastinal involvement (3a 4a) in two cases on DLPD and considerable improvement after one course of PVP (3b, 4b).

pressive activity [26 27] In this clinical material myelosuppression was never severe, except for patients with marked lymphoid infiltration of the bone marrow In the remaining cases chemotherapy was almost perfectly tolerated. Bone marrow biopsies showed frequent aspects of dyserythropoiesis, with intercytoplasmic bridges and megaloblastoid transfor



mation of the erythroid precursor In 35% of the cases VCR had to be discontinued because of neurotoxicity the drug was then replaced with vinblastine.

Discussion

Survival in advanced lymphomatous disease has been powerfully influenced by the recent introduction of active combination chemotherapy



Fig 3 4 Serial chest radiographs, showing mediastinal involvement (3a, 4a) in two cases on DLPD and considerable improvement after one course of PVP (3b, 4b).

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Discussion

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Table VII Various regimes employed in NHL

Authors	Protocol	Histology	CR rates %	PR rates %
HOOGSTRATEN *et al.* [18]	COP	DLPD	31	69
		DHL	30	53
LOWENBRAUN [4]	MOPP	DLPD	46	33
		DHL	37	35
LUCE *et al* [25]	COP	DLPD	50	–
		DHL	39	–
BONADONNA *et al* [3]	ABP	DLPD	44	–
		DHL	39	–
DE VITA *et al* [10]	C MOPP	DHL	41	29
BERD *et al* [2]	COMA	DHL	60	40
CARELLA [6]	PVP	DLPD	56	26
		DHL	55	30

COP = Cyclophosphamide, vincristine, prednisone MOPP = nitrogen mustard vincristine, procarbazine prednisone ABP = adriamycin bleomycin prednisone C MOPP = cyclophosphamide, interchangeably with nitrogen mustard in MOPP COMA = cyclophosphamide, vincristine, methotrexate, cytosine arabinoside, and PVP = peptichemio, vincristine prednisolone.

schedules. However notwithstanding single favourable evaluations, prognosis is much poorer in NHL in comparison with Hodgkin s disease [3 4 11 16 30 31] Although JOHNSON [19] has recently reported encouraging results employing total body irradiation in advanced stages of DLPD-NHL, chemotherapy is still generally preferred in NHL with systemic dissemination [30] In addition, results obtainable with chemotherapy appear to be dependent on the histologic type of the lymphoma, since it is generally agreed that nodular lymphomas have a better prognosis than the diffuse forms.

An analysis of 405 patients at the Stanford University has shown that 44% of NHL were nodular in type [20] and that the nodular pattern was associated with longer survival when compared with the diffuse one. One may suspect, accordingly that many long term survivals reported in the literature after single or combination chemotherapy in so-called lymphosarcomas and reticulum cell sarcomas may have occurred in patients with nodular NHL.

Since the first demonstration of better results of combination versus single-drug regimens [21] various associations have been proposed for NHL, e.g. the widely used COP regimen (cyclophosphamide, vincristine and prednisolone) is capable of inducing CR rates ranging from 50–55% in DLPD to 35–40% in DHL [1 17 18 25 36] Cyclophosphamide (CY) has also been employed in other combinations for NHL as summarized in table VII [2, 10, 15 22, 35 36]

The purpose of this paper is to demonstrate that the replacement of CY with PTC in the classical COP regimen (thus changed in PVP) is capable of inducing results which are at least comparable to the correspondent obtained with the COP protocol. Besides the results obtained in previously untreated patients, it should be stressed that 14 cases, previously treated with combinations including CY and subsequently relapsed, showed a remission rate of 64 / (CR+PR) when treated with the PVP regimen, thus demonstrating that PTC can still induce remissions in CY-resistant patients. Recent investigations, performed in the laboratories where PTC was synthesized, have shown a marked antimetabolic effect of the drug, probably due to the peptidic sequences bound to the central alkylating group and possibly responsible for PTC's greater chemotherapeutical effect [12]

In conclusion, according to our results, it would appear that the PVP protocol in diffuse NHL can (a) induce remission rates comparable if not higher than with other combinations, (b) cause dramatic improvement in oncologic emergencies, (c) induce remissions in CY-refractory patients, and (d) ensure good therapeutic effects with low or moderate toxicity

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Inherited Platelet Abnormalities Associated with Low Factor VIII Activity in the Same Family

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Key Words. Bleeding disorders · Factor VIII deficiency · Haemophilia · Platelet disorders · Thrombopathies

Abstract. Six of eight examined members belonging to two generations of the same (NEO-TUR) family were shown to have functional changes in platelets and/or moderate decrease of factor VIII activity (FVIII:C) in plasma, with normal values of factor VIII-related antigen (VIII R:AG). Platelet defects (mainly reduced PF 3 availability present in five patients) and factor VIII decrease were combined differently in individual members. Only two male members with both the PF 3 and FVIII:C defects had moderate haemorrhagic symptoms following traumatic injuries. One of them had also an absent adhesiveness to glass, the other one an absent adhesiveness to collagen and reduced platelet aggregation by ADP and by collagen. Bleeding time, platelet function tests (in the other members) and routine coagulation tests were within normal range; ristocetin aggregation was also normal in all members. We think that two inherited defects, mild haemophilia A and 'sui generis' thrombocytopathy co-exist in this family.

The observation of a patient with a mild haemorrhagic syndrome, in which a functional platelet defect was associated with a reduced level of factor VIII activity in plasma, led us to investigate his family in which variously combined defects of platelet function and of coagulation were found.

Clinical Data

A total of eight members from two generations of the same family (NEO-TUR) were examined (fig. 1). Some subjects (cases I-7, I-9, II-7) were repeatedly examined over a period of about 1 year; the other members had only one examination.

The authors are indebted to Dr. T. Barbui for having performed immunological assays of F VIII R:AG.

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Assays of clotting factors V VIII, IX, X, XI, XII were carried out with one-stage methods (reagents and substrates from Warner Lambert). Factor XIII was determined with SIGG and DUCKERT's [14] monoiodoacetate tolerance test. Factor VIII-related protein was immunologically evaluated with LAURELL's [11] technique using Behringwerke antiserum. For bleeding time the techniques of both Ivy and Duke were applied.

Platelet counts were performed with the method of BRECHER and CRONKITE [3]. Platelet adhesiveness to glass was studied with the method of SALZMAN [15] ('Adeplat' kit, Mascia Brunelli, Milano, Italy), adhesiveness to collagen (Collagen, Stago, Asnières, France) following WEISS et al. [20], and in vivo adhesiveness following BORCHGREVINK [1]. Aggregateur' reagents (Stago) were utilized for platelet aggregation induced by ADP (2–4 μM, final concentration), adrenaline (2 μM, final concentration) and collagen; ristocetin (Lundbeck, Copenhagen, Denmark) was used at a final concentration of 1.0 mg/ml [10]. An EEL (model 169) aggregometer with a Philips direct writing recorder was used in all procedures.

Platelet factor 3 (PF3) availability was determined following SPAET and CINTRON [15] on citrated platelet-rich plasma, in basal conditions and after adding ADP to PRP or on platelet lysates obtained by freezing and drying. Platelet factor 4 (PF4) was assayed with the method of HARADA and ZUCKER [8]. Clot retraction was studied with the method of CAEN et al. [4].

Fibrin polymerization was recorded on an Elvi 670 photometer with the method of FERRY and MORRISON [7].

Results

The most significant data are summarized in table I. One female member (case II–8) gave completely normal results, and another female member (case I–5) was also normal, apart from a moderate thrombocytopenia with a slightly prolonged bleeding time. Five of the remaining six members (4 males and 1 female) were found to have a defect in the PF 3 availability with a complete or almost complete absence in three of them (case I–9 II–6, and II–7), and reduced in the remaining two cases (I–2 and I–7). Normal amounts of PF 3 were, however detectable in all these cases after addition of ADP to platelet-rich plasma as well as after subjecting PRP to freezing and drying. Moreover in two of these cases (both male) a moderate reduction of plasma factor VIII coagulant activity (FVIII:C) was found (25–30%, in case II–7 and 15–20% in case I–9). The sixth affected member, still of male sex (case I–1), had only the latter defect (F VIII:C = 30%).

Platelet adhesiveness and aggregation were found normal in all cases, with two exceptions. The propositus (case II–7) showed absence of adhesiveness to collagen, with reduced aggregation both by ADP and by colla-

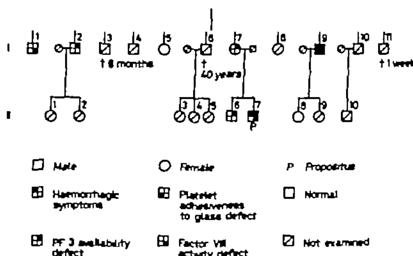


Fig. 1. Pedigree of the NEG-TUR family

Only two of the tested members had haemorrhagic symptoms. The propositus (case II–7), an 18-year-old male, had experienced several haemorrhages, always after trauma (the first time, when he was 12, following tonsillectomy· and later twice, after dental extraction). On one occasion he required blood transfusions before bleeding could be stopped. The patient also referred posttraumatic suffusions and haematomas out of proportion to the trauma. The second patient with a haemorrhagic history (case I–9), a paternal uncle of the propositus, had suffered from epistaxis in his childhood and from prolonged bleeding after dental extractions.

Two more male members of the family had also a history of mild, posttraumatic haemorrhagic syndrome, similar to that just mentioned, but neither of them could be examined one (case I–6) died in 1964 because of an unrestrainable haematemesis *in a few weeks after a gastrectomy for gastric ulcer* the other (case I–4) refused examination. Finally two members of the first generation (case I–8 and I 10) live abroad with their families and, therefore, could not be examined.

Methods

Routine clotting tests, performed on oxalated plasma, included activated partial thromboplastin time (PTT reagent, Behringwerke AG Marburg, Germany· normal value <55 sec), Howell's recalcification time, prothrombin time (Calcium thromboplastin, Behringwerke), prothrombin in serum 2 h after clotting (Fibrinogen, Warner Lambert, Morris Plains, NJ USA Calcium thromboplastin, Behringwerke; normal value <20%), plasma fibrinogen level (Gram's ponderal method), thrombelastogram (Hartert's thrombelastograph).

Assays of clotting factors V VIII, IX, X, XI, XII were carried out with one-stage methods (reagents and substrates from Warner Lambert). Factor XIII was determined with Sboo and Duckert's [14] monoiodoacetate tolerance test. Factor VIII-related protein was immunologically evaluated with Laurell's [11] technique using Behringwerke antiserum. For bleeding time the techniques of both Ivy and Duke were applied.

Platelet counts were performed with the method of Brecher and Cronkite [3]. Platelet adhesiveness to glass was studied with the method of Salzman [13] ('Adeplat' kit, Mascia e Brunelli, Milano, Italy), adhesiveness to collagen (Collagen, Stago, Asnières, France) following Weiss *et al.* [20], and *in vivo* adhesiveness following Borchgrevink [1]. Aggregateur' reagents (Stago) were utilized for platelet aggregation induced by ADP (2–4 µM, final concentration), adrenaline (2 µM, final concentration) and collagen; ristocetin (Lundbeck, Copenhagen, Denmark) was used at final concentration of 1.0 mg/ml [10]. An EEL (model 169) aggregometer with Philips direct writing recorder was used in all procedures.

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Fibrin polymerization was recorded on an Elvi 670 photometer with the method of Perry and Morrison [7].

Results

The most significant data are summarized in table I. One female member (case II–8) gave completely normal results, and another female member (case I–5) was also normal, apart from a moderate thrombocytopenia with a slightly prolonged bleeding time. Five of the remaining six members (4 males and 1 female) were found to have a defect in the PF 3 availability with a complete or almost complete absence in three of them (case I–9 II–6, and II–7), and reduced in the remaining two cases (I–2 and I–7). Normal amounts of PF 3 were, however detectable in all these cases after addition of ADP to platelet-rich plasma as well as after subjecting PRP to freezing and drying. Moreover in two of these cases (both male) a moderate reduction of plasma factor VIII coagulant activity (FVIII:C) was found (25–30% in case II–7 and 15–20% in case I–9). The sixth affected member still of male sex (case I–1), had only the latter defect (F VIII:C = 30%).

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Table I Platelet function and coagulation tests in members of the NEG-TUR family (the most significant results are reported)

Patient No. sex	PF 3 availability	Platelet adhesiveness		Factor VIII, %		PTT sec	Serum prothrombin %	Notes
		glass	collagen	AHF	antigen			
I-1 M	normal	normal	normal	31	–	57	15	–
I-2, M	moderately reduced	normal	–	100	–	42	12	–
I-5 F	normal	normal	–	100	–	32	21	mild thrombocytopenia (80,000/µl) bleeding time 7 min
I-7 F	reduced	normal	normal	80	190	45	8	F VIII coagulant/antigen ratio 0.42
I-9 M	severely reduced	absent	normal	15–20	125	58	46	*in vivo* platelet adhesiveness [1] 9 %
II-6, M	absent	normal	normal	90	–	36	18	son of case I 7
II-7 M (propositus)	absent	normal	absent	25–30	108	64	34	son of case I-7 aggregation by ADP 24-35 % by collagen 76-45
II-8, F	normal	normal	normal	130	–	39	7	daughter of case I-9

gen, but without any trend to disaggregation. A second male member of the family (case I-9) was found to have a marked decrease of adhesiveness to glass and in the *in vivo* test of BORCHGREVINK [1] (9%) but with a normal platelet aggregation induced by ADP and collagen. Platelet count, size and morphology clot retraction and bleeding time were also consistently normal in all affected members.

In the three subjects with reduced F VIII C, platelets were found to aggregate normally with ristocetin while immunoassays demonstrated normal amounts of F VIII R.AG in plasma of examined cases (I-9 II-7 I-7).

Finally routine coagulation tests were normal in all members of the family with only two exceptions a slightly prolonged aPTT was seen in the three subjects with reduced F VIII C and a decreased prothrombin consumption was found in the two patients with associated defects of PF3 and F VIII C, while the test was normal in the members with only one isolated defect (i.e. PF 3 or F VIII C)

Thrombelastographic parameters were generally normal, but two subjects (case I–7 and II–7) had moderately reduced values of maximal amplitude, a finding of difficult interpretation since both factor XIII and fibrin polymerization (apart from fibrinogen and platelet count) were normal. Mild haemorrhages, only after traumas (such as minor surgery or teeth extraction) were present only in the two patients in which both defects (in plasma and in platelets) were associated.

Comment

The association of functional platelet abnormalities with a decreased plasma F VIII·C, observed in 2 of 8 examined members of the family raises the question of a possible diagnosis of von Willebrand's syndrome, with particular regard to case I–9 in which a defect in platelet adhesiveness to glass was found. Even this patient, however did not fall within the known variants of the disease, since he had normal bleeding time, his platelets were normally aggregated by ristocetin, his plasma level of F VIII R.AG was normal. Moreover the response of plasma F VIII.C level to infusion of F VIII containing material is one of the 'haemophilic' type, i.e. with an immediate steep rise followed by a relatively rapid decrease. Finally an impaired availability of PF 3 such as that seen in our case, does not fall within the common findings in von Willebrand's syndrome. Even more so, this diagnosis must be excluded for the second of our cases with associated defect of platelet function and of factor VIII, the propositus, who, apart from the same features already mentioned, also had normal platelet adhesiveness to glass.

In the other affected members of the family only a single defect is present, i.e. the reduction of F VIII:C (case I–1) or the impaired PF 3 availability (case I–2, I–7 and II–6), the latter being the most frequent defect detectable in the family (5 of 6 affected members) From this point of view the disease could be identified as a hereditary functional platelet disorder of the type of 'thrombopathy' in the sense proposed 10 years ago by Quick [12] More recent studies, however and particularly those of Holmsen and Weiss [9] Weiss [18] and Weiss and Rogers [19] showed that a more complex impairment of platelet function was often present in patients with defect of PF 3 availability namely a reduced release of platelet ADP either because of an actual reduction of the 'storage pool' or due to a damage of releasing mechanisms. The most frequent and impor

tant features of this complex disorder are missing in all our cases, apart from a reduced adhesiveness to glass, present only in case I–9 and a reduced aggregation by ADP and collagen inconstantly found in case II–7

In some male members of the family however a plasmatic defect exists, associated or not with the platelet disorder a moderate decrease of FVIII C activity while F VIII R.AG is within normal range. This finding, and the limitation to only male members of the factor VIII defect, suggests haemophilia. Moreover the female patient I–7 most probably a carrier (being sister of two affected males and mother of the third), shows a typical pattern of a haemophilia carrier a biological activity of factor VIII of 80%, but a very low F VIII C/F VIII R.AG ratio, close to 0.5 Finally the response of F VIII C activity in two of the affected members to infusion of plasma shows a pattern typical of haemophiliacs.

The most reliable hypothesis is that in the family studied by us a functional platelet defect – most frequently a reduced PF 3 availability – is associated by chance with a mild haemophilia. The association of a platelet abnormality with a coagulopathy in the same family has rarely been reported in the literature and the NEG TUR family does not seem to fit into any of the known variants. In the two families described by CHESNEY *et al* [5] and in the cases observed by CROWELL and EISNER [6] there was an association of thrombocytopathy (defective release, reduced adhesiveness, long bleeding time) with haemophilia A. One of the cases reported by WEISS [17] presented an association of thrombocytopathy with reduced PTA (factor XI) The family BU ST studied by BOWIE *et al.* [2] showed a decreased F VIII·C with a reduced PF3 Other associated features, however induced the authors to include these cases among the variants of von Willebrand s disease. ULUTIN [16] reported some cases with defective PF 3 associated with reduced F VIII C, classified as 'thrombopathia haemophiliaca

Apart from these difficulties in the classification, some members of the NEG TUR family presented laboratory data of a difficult explanation (such as the low values of ma in thrombelastographic tracings of cases I–7 and II–7) or with some discrepancy between them (as the absent adhesiveness to collagen with only a reduced aggregation to collagen in case II–7)

To conclude, in the field of hereditary functional platelet disorders, besides well-known diseases, such as Glanzmann s thrombasthenia, Bernard-Soulier's and von Willebrand's syndromes, other groups of thrombocytopathies are being progressively defined with the advance of investi

gational techniques. The recognition of different associations (such as those here reported) and of variants with regard to typical patterns, however makes systematic classification of these syndromes still open to and requiring further research.

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Addendum. Two more members of the family (I–10 and II–10), usually living abroad, were examined during the printing of this paper Both showed normal values of F VIII:C, PF 3 availability and platelet adhesiveness.

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Chemotherapeutic Remissions in Wistar Furth Rat Acute Myelogenous Leukemia: A Model for Human AML[1]

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Key Words. Acute myelogenous leukemia Chemotherapy of AML Leukemia Rat leukemia

Abstract. Acute myelogenous leukemia (AML) of the inbred Wistar/Furth (W/Fu) rat is pathophysiologically similar to human AML. Subcutaneous transplantation of 1.0×10^6 cells of clonal tissue culture line of W/F AML into 6- to 8-week-old rats produced local myeloblastomas in 8–10 days which progressed to infiltration of regional nodes, replacement of >90% of the bone marrow ascites, and fatal peripheral blood leukemia with concomitant hyperlysozymemia. Single doses of adriamycin, daunomycin, actinomycin, cytosine arabinoside, or Cytoxan in rats with 1.0 cm myeloblastomas produced complete tumor regression while busulfan, vinblastine, vincristine, dexamethasone, and Methotrexate were relatively ineffective. Responses were associated with delay in progression to peripheral blood leukemia and prolonged survival. Similar results were obtained following treatment of rats with already disseminated leukemia. The demonstration of response to drugs known active against human AML indicates that the W/Fu AML should be valuable model for rapid evaluation of new chemotherapeutic agents for clinical use.

Acute myelogenous leukemia (AML) is a fatal disease in man. Chemotherapy has been the major approach in treatment [3 9 14 18, 22, 24 26, 27 29–31], however even with current drug protocols, complete remission rates of only 70% can be achieved and the median survival of responders is discouragingly low at 3–19 months [3 9 18, 26 27 31]

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Immunotherapeutic [17] and radiotherapeutic [23] approaches have met with little success and recent attempts at bone marrow transplantation following total body irradiation are still in the experimental stages [25]

The development of new therapeutic approaches in AML has been hampered by the lack of an experimental model. While several animals AMLs have been described [15 20 21] none has been suitable for experimental chemotherapy owing to poor transplantability or inability to establish tissue culture lines for study of cellular kinetics, morphology and homogeneity AML in the rat closely mimics human AML [15] but is rare in the inbred strains wherein transplantation studies are possible. Recently a transplantable rat leukemia of the inbred Wistar/Furth (W/Fu) strain has been established as a continuous clonal line *in vitro* and shown to be similar to human AML in biological and cytological properties including the formation of solid tumors, induction of hyperlysozymemia with lysozymuria *in vivo* [12] and demonstration of stable myeloblast morphology with continuous production of lysozyme *in vitro* [11]

In the present report, tests of a variety of chemotherapeutic agents in W/Fu AML-revealed activity of those known active in human AML.

Materials and Methods

Animals 6- to 8-week-old weanling W/Fu rats (Charles River Breeding Laboratories, Boston, Mass.) were housed two per cage and fed standard laboratory chow

Leukemia cells. W/Fu AML Cl 2 [12] was repassaged *in vivo* for 2 months intraperitoneally to weanling W/Fu rats and uniformly produced leukemia and ascites in animals prior to death. Ascitic fluid samples uniformly gave leukemia cell viability of >85% by trypan blue exclusion.

Tumor induction. For the quantitation of rapid action of chemotherapeutic agents, unifocal solid tumors were induced by subcutaneous infrascapular inoculation of 1×10^{6} W/Fu AML ascitic fluid cells in 0.5 ml of normal saline via 25-gauge needles. Rats with tumors of 1.0 cm in diameter as measured by taking the average of four diameters with a standard tumor calipers were considered ready for chemotherapy Evidence of concomitant peripheral blood leukemia or ascites excluded rats from study

Disseminated leukemia was induced in other groups by i.v inoculation of 1.0×10^{6} W/Fu AML ascitic fluid cells in 0.5 ml of normal saline. Peripheral blood WBC counts and differential counts were performed on daily tail vein venous blood samples. Rats were considered ready for chemotherapy on the day of appearance of W/Fu AML blast cells representing $\geq$10% of the peripheral blood WBC count.

Chemotherapy Adriamycin, daunomycin, actinomycin, dexamethasone, cyclophosphamide (Cytoxan®), vincristine (Oncovin®), vinblastine (Velban®), and

amethopterin (Methotrexate®) were administered intravenously to groups of at least 10 ether-anesthetized rats per drug dose via the surgically exposed jugular vein. Extravasation of the drug during administration excluded the animal from further study. Cytosine arabinoside (Ara-C®) was administered subcutaneously in three divided injections over 24 h. Busulfan was given by gastric tube in a solution of 0.9% NaCl. Doses of each drug were individualized to mg/kg body weight. Control groups received no drugs. Rats were observed for spontaneous hemorrhage and clinical signs of anemia. All had daily WBC and differential counts, hematocrits and platelet counts. Tumor diameter was measured daily and rats were weighed and physically examined for the appearance of enlarged regional lymph nodes, ascites or organomegaly. Complete response was defined as absence of palpable disease in the s.c. passage group and disappearance of PB blast cells in the i.v. passage groups. Partial response was defined as >50% decrease in the above parameters, respectively.

Pathological examination. After gross pathologic examination, histopathological sections were prepared from liver, spleen, bone marrow, lymph node, and local tumors of leukemic and control animals by standard procedures.

Results

The natural course of the disease induced following s.c. or i.v. inoculation of 1.0×10^6 cells was first studied. 100% of rats receiving s.c. W/Fu AML cells developed 1.0-cm tumors at the injection site by 14 days, with a median time of 8 days. While 100% and 20% subsequently demonstrated local lymph node disease and ascites, respectively, this occurred later with a median time of 18 and 20 days, respectively, after inoculation. Hepatosplenomegaly and hind limb paralysis also occurred later with a median of 18 and 19 days, respectively. All animals were dead by 32 days with a median survival of 28 days following inoculation. Autopsy examination revealed local s.c. tumor progression in all rats with more frequent visceral involvement than that detected clinically. Meningeal leukemia was detected in 8% of autopsied rats, and pulmonary leukemia cell infiltrates in 75%. While 100% of rats had postmortem bone marrow involvement with W/Fu cells, there was no clinical or hematologic evidence of marrow depression causing detectable leukopenia, anemia, or thrombocytopenia. Furthermore, when other rats with localized 1.0-cm s.c. tumors were sacrificed at days 8–10 in an attempt to detect subclinical microscopic spread of tumor, no gross visceral or marrow involvement was observed. Since all rats had peripheral blood leukemia between 17 and 23 days, the data indicate that detectable hematogenous dissemination from the s.c. inoculation site occurred later in the course of the disease.

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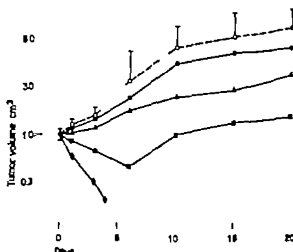


Fig 1 Kinetics of response to a single i.v dose of adriamycin. W/Fu rats with 1 0 cm AML solid s.c. tumors received on day 0· 0.5 mg/kg (●), 5.0 mg/kg (▲), 10.0 mg/kg (■) 20.0 mg/kg (♦) or no treatment (O) The tumor volume was measured daily as described in Materials and Methods. Results represent the mean volume for each group of at least 10 animals with SD bars included on untreated rats only

Rats inoculated with 1 0×10⁶ cells i.v developed systemic disease in 7–12 days with a median of 8 days, and all were dead in a median time of 8 additional days with postmortem findings similar to the s.c. passaged group

Chemotherapy of W/Fu AML myeloblastomas The effect of single i.v doses of drugs on rats with subcutaneous 1 0 cm myeloblastomas was first investigated. Rats receiving doses up to neurotoxic levels of 1 0 mg/kg vincristine or vinblastine, doses of up to 50 mg/kg busulfan, or doses of up to 10 mg/kg dexamethasone demonstrated little evidence of tumor regression compared to the control group 50% of the rats receiving Methotrexate (100 mg/kg) showed partial response with some responses detectable as early as 1 day after chemotherapy Higher Methotrexate doses were lethal. In contrast, rats receiving adriamycin, daunomycin actinomycin, cytosine arabinoside, or Cytoxan demonstrated complete responses in 50–100% of the animals inoculated with sulethal doses. High-dose groups receiving 10 or 20 mg/kg adriamycin, 10 mg/kg daunomycin, 1 mg/kg actinomycin, or 150 mg/kg Cytoxan demonstrated fatal marrow or gastrointestinal toxicity in all complete responders. While complete responses of

up to 90 days were obtained, all relapsed and died with disseminated AML.

Chemotherapeutic responses of rats with i.v. passaged W/Fu AML and disseminated leukemia were similar for each drug tested, however, rapid relapse following single doses prevented evaluation of relative effectiveness. An example of the ease of quantitation of dose-related tumor regression following single doses of i.v. chemotherapy is shown for adriamycin in figure 1.

Toxicity of chemotherapy in W/Fu AML. Myelosuppression was directly dose-related in rats receiving single dose adriamycin, daunomycin, or Cytoxan with leukopenia detected as early as 3 days after drug and persisting until day 12. Rats receiving multiple low doses of these drugs in other experiments had reduced leukopenia and thrombocytopenia, with comparable tumor reduction. Rats in the fatal high-dose adriamycin and daunomycin groups lost weight progressively with postmortem evidence of mucosal ulcerations throughout the bowel. Cytoxan-treated groups demonstrated dose-related weight loss with no fatal toxicity. Cytosine arabinoside was nontoxic in doses which produced tumor responses and increases in survival comparable to toxic doses of adriamycin or daunomycin.

Discussion

The need for a 'predictive' model [6] of human AML has been noted to be one of the more important issues in hematologic experimental chemotherapy [15]. Evaluation of new chemotherapeutic drugs is commonly carried out in well-known experimental tumor systems [2, 4, 5, 7, 8, 13, 19, 32] which may be generally predictive of antineoplastic effect, however, data obtained with a specific tumor model may be of further aid to the clinician designing therapy schedules for each specific human malignancy. No specific model exists for human AML, and there is a discouraging absence of response in man to some drugs which appear effective in the L1210 or AKR mouse leukemia.

In the present study a transplantable AML of the inbred W/Fu rat was evaluated as a therapeutic model for human AML. While all rats with untreated 1.0 cm subcutaneous myeloblastomas died with progression of disease to bone marrow, ascites, and organ infiltration, a predictable 10-day interval prior to progression was used to assess relative cytoreduction of a large local tumor burden. Drug administration for all agents except

cytosine arabinoside and busulfan was carried out via the intravenous route. Adriamycin daunomycin, actinomycin, cytosine arabinoside and Cytoxan were each shown to be effective in single doses. Time to leukemic dissemination and survival were increased in all complete responders. Rats in all treatment groups relapsed and died with disseminated AML. Rats with selectively induced systemic disease by i.v passage of tumor cells demonstrated a trend toward similar dose-related increase in survival with each active drug, however the relative rates of cytoreduction could not be easily measured.

Bone marrow and gastrointestinal toxicities, as evidenced by depressed WBC and platelet counts and bowel ulceration, were dose-related in adriamycin and daunomycin groups and were comparable to those previously determined in the rat [16 33] Nephrotoxic and cardiotoxic effects were not directly evaluated although high-dose adriamycin groups received levels previously determined to be cardiotoxic [16, 33]

One explanation for the poor clinical chemotherapeutic results obtained in human AML compared to acute lymphatic leukemia may be inapparent but systemically inadequate cytoreduction. An explanation of this phenomenon may be the presence of unknown 'sanctuary sites or simply drug resistance. To help resolve this dilemma, the W/Fu AML has several advantages as a specific model. Easily measurable local tumors should facilitate development of schedules of multifractionated chemotherapy [7] using drugs with a rapid plasma half life [32] with comparison to multifractionated radiation therapy [28] The pathophysiology of progressive untreated W/Fu AML is very similar to human AML including the detection of meningeal leukemia in 8% of autopsied rats. Furthermore, postchemotherapy relapse is associated with a high 93.3% incidence of CNS involvement [manuscript in preparation] similar in neuroanatomic site to that recently reported in BD-IX rats with the L5222 leukemia [1] a nonmyeloid leukemia [HOELZER, personal commun.] The W/Fu AML is a weakly antigenic leukemia derived in an inbred strain with an LD_{50} of 5×10^{2} multiply passaged cells, and demonstrates no detectable type C virus production *in vivo* [12] Thus, evaluations of cure with the W/Fu ML avoid both the mechanism of rejection of residual but immunogenic viable cells which by analogy might produce relapse in the individual patient and the added immunogenicity of virus-producing cells [2, 10] The low LD_{50} of the W/Fu AML should permit study of relapse in rats receiving therapeutic bone marrow transplantation following combination chemotherapy and total body irradiation since current clinical

failures of this program are commonly due to recurrence of AML [25]. Complete responses of W/Fu AML following treatment with human AML – active drugs indicate that this model may be a valuable test system for new clinical chemotherapeutic agents. These studies are in progress.

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Influence of Cytostatics on Some Platelet Functions in vitro

V Hydroxyurea

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Key Words Cytotoxic drugs Hydroxyurea Platelet functions Tumor chemotherapy

Abstract Hydroxyurea added to citrated platelet-rich plasma *in vitro* did not influence the aggregation of the platelets, their adhesion to glass, their release of aggregating activity platelet factor 4 or availability of platelet factor 3 Neither did it have any effect on the uptake of ¹⁴C-serotonin, the reptilase clot retraction or the coagulation system.

Hydroxyurea (Hydrea® Squibb) is a cytostatic which probably exerts its effect by disturbing the synthesis of DNA [3] It is sometimes used alone, but more often combined with other drugs in the treatment of many forms of hemoblastosis [2, 3 13] It has been shown that rubidomycin [9] and alkeran [5] have a direct effect on the platelets *in vitro* while cytosine arabinoside [10] and peptichemio [4] have not

The aim of the present investigation was to find out whether hydroxyurea has any deleterious effect on platelet function *in vitro*

Material and Methods

Blood samples were obtained from healthy volunteers by allowing 9 parts of venous blood to flow directly into test tubes containing 1 part of 0.11 mol trisodium citrate

Platelet-rich plasma (PRP) was obtained by centrifuging the blood at 280 g for 20 min at 16 C. The platelet count was adjusted to 300,000/ l by addition of platelet-poor plasma (PPP) from the same volunteer PPP was prepared by centrifuging PRP at 12,000 g for 20 min at 12 C.

Table I. Effect of hydroxyurea on some platelet parameters (means and standard deviations of 8 experiments)

	Aggregation			Serum aggregating activity		Adhesion to glass slide	Uptake of serotonin
	ADP %	adrenaline %	collagen %	5 min %	30 min %	arbitrary units	%
Buffer	54±3	43±14	52±8	30±7	19±10	30±1	82±7
Hydroxyurea 0.33 mmol/l	4.±12	32±19	45±7	28±4	16±7	30±2	80±9
Hydroxyurea 0.65 mmol/l	51±5	27±17	46±9	25±7	16±10	26±2	71±19

Percentage of light transmission after 4 min

Tested 5 and 30 min after coagulation of the PRP with thrombin. The figures give the light transmission in percent as recorded after 4 min.

Collagen (Stago, Asnières, France), ADP (Sigma), and adrenaline (Spofa, Prague, ČSSR) were used and diluted to desired concentration by addition of Michaelis buffer at pH 7.35

Hydroxyurea (Hydrea Squibb) was dissolved to desired concentration in Michaelis buffer at pH 7.35

The availability of platelet factor 3 platelet aggregation and release of platelet factor 4 (PF 4) were estimated by methods described in details elsewhere [7].

The adhesion of platelets to glass slide was assessed with the use of a method by MASON *et al.* [11] as modified by KOVÁČOVÁ and KUBISZ [6].

The uptake of ¹⁴C-serotonin and retraction of reptilase clots wer determined according to KUBISZ and SUBANOVÁ [8]

The release of serum aggregating activity (AA) was measured in accordance with CAEN *et al.* [1].

The Quick time, cephalin-kaolin time, reptilase time, thrombin time and fibrinolytic activity in euglobulin clots were determined according to NILSSON [12].

Results

Platelet aggregation Hydroxyurea in a concentration of 0.33–0.65 mol had no effect on the aggregation induced by ADP (4 μmol), adrenaline (6 mol) or collagen (20 mg/l table I).

Retention of platelets to a glass slide No difference was found between the retention before and after addition of hydroxyurea (table I).

Table II Effect of hydroxyurea on the platelet factor 3 availability recalcification time in seconds (means and standard deviations of 8 experiments)

	Incubation time min	ADP 4 µmol/l sec	Adrenaline 6 µmol/l sec	Collagen 20 mg/l sec
PRP + buffer	0	37.5±4	42.6±4	43.4±5
	10	16.4±1	18.1±3	16.8±1
	15	16.2±1	17.0±3	16.1±1
PRP + hydroxyurea 0.33 mmol/l	0	36.5±4	39.6±4	39.0±3
	10	18.7±3	16.8±2	17._±1
	15	18.6±3	16.5±1	17.1±
PRP + hydroxyurea 0.65 mmol/l	0	38.6±4	40.8±4	39.1±6
	10	19.1±2	19.4±3	16.7±1
	15	18.9±3	18.4±1	16 ±2

Table III Percentage PF4 released after incubation for 0 5 and 10 min with different inducers (means and standard deviations of 8 experiments)

	Incubation time min	ADP 4 µmol/l	Adrenaline 6 µmol/l	Collagen 20 mg/l
PRP + buffer	0	8±2	7±	8±
	5	43±11	44±10	50±9
	10	51±11	54±9	60±6
PRP + hydroxyurea 0.33 mmol/l	0	6±2	6±	5±2
	5	25±12	30±13	46±5
	10	35±12	48±4	51±3
PRP + hydroxyurea 0.65 mmol/l	0	6±	6±	5±2
	5	1±11	25±13	45±7
	10	30±14	39±9	50±4

Uptake of ^{14}*C serotonin* was not diminished by hydroxyurea (table I)

Release reaction Release of serum AA platelet factor 4 and availability of PF 3 were not influenced by hydroxyurea (table I–III)

Reptilase clot retraction was not affected by hydroxyurea (table IV)

Coagulation system The addition of hydroxyurea had no effect on the Quick time cephalin kaolin time, reptilase time thrombin time and the fibrinolytic activity of euglobulin clots (table V)

Table IV Effect of hydroxyurea on reptilase clot retraction

	Clot retraction min	ADP %	Adrenaline %	Collagen [illegible]
PRP + buffer	15	41±10	41±13	36±12
	30	68±9	71±10	63±9
	45	71±9	75±9	73±8
PRP + hydroxyurea 0.33 mmol/l	15	58±10	53±10	56±8
	30	74±8	80±8	81±7
	45	80±7	83±9	85±7
PRP + hydroxyurea 0.65 mmol/l	15	35±11	23±17	21±12
	30	73±10	68±12	61±11
	45	81±9	80±10	81±9

PRP was incubated with buffer or hydroxyurea for 10 min at 37°C. The platelets wer activated by ADP (0.1 mmol/l), adrenaline (1 mmol/l) or collagen (50 mg/l). Immediately afterwards reptilase was added and the volume of fluid separated from the clot after 15, 30 and 45 min was expressed as percentage of total (means and standard deviations of 5 experiments)

Table V Effect of hydroxyurea on some coagulation parameters (means and standard deviations of 8 experiments)

	Quick time sec	Cephalin-kaolin time sec	Reptilase time sec	Thrombin time sec	Euglobulin clot lysis time h
PRP + buffer	14.2±1.0	44.6±0	130±26	19.1±0.1	~3
PRP + hydroxyurea 0.33 mmol/l	15.2±1.4	48.3±[illegible]0	127±17	19.7±0.1	3
PRP + hydroxyurea 0.65 mmol/l	15.7±1.8	49.8±1.9	142±13	20.4±0.1	3

Discussion

Judging from the present investigation, addition of hydroxyurea does not influence the platelet function *in vitro*. This is in agreement with the conception that hydroxyurea attacks the DNA synthesis, which is of little

of no importance to the platelets. Since the concentrations of hydroxyurea used in this study are similar to those used in the treatment of patients, these *in vitro* studies suggest that hydroxyurea might be a useful drug when a patient with bleeding symptoms has to be treated with cytostatics. It must however first be checked whether these findings *in vitro* can also be made in corresponding administration *in vivo* since it cannot be excluded that hydroxyurea might exert a harmful effect on the megakaryocytes with the formation of defective platelets as a result.

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Scanning Electron Microscopic Study of Leukemic Human B Lymphocytes

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Key Words. B lymphocytes · Cell surface · Hairy cell leukemia · Leukemia · Malignant lymphomas · Scanning electron microscopy

Abstract. Peripheral blood lymphocytes from patients with chronic lymphocytic leukemia (CLL), lymphoplasmacytoid lymphoma, centrocytic lymphoma and hairy cell leukemia were studied by scanning electron microscopy (SEM). In general, SEM revealed rather homogeneous cell populations. Most lymphocytes displayed moderately villous surface architecture, although smooth surfaces predominated in 3 cases with CLL and in 1 case with lymphoplasmacytoid lymphoma. Hairy cells showed surface features of both lymphocytes and monocytes. The results indicate that leukemic B and T lymphocytes cannot be distinguished by SEM alone.

Considerable interest in morphological studies of human lymphocytes has been generated by the recognition of two functionally distinct populations, the thymus-dependent (T) and the bone-marrow-dependent (B) lymphocytes. Although both cell types are characterized by certain surface receptors and membrane markers [1, 2], no significant differences have been observed in their ultrastructural features using transmission electron microscopy [12]. Recent findings indicate that normal human T and B lymphocytes can be distinguished by scanning electron microscopy which provides a three-dimensional view of details on the cell surface [25, 27]. Thus, T cells were found to be usually smooth and to display only few short and stub-like microvilli, whereas most B cells are characterized by a large number of long, finger like projections. In the present study SEM has been applied to analyze the surface morphology of leukemic human lymphocytes bearing surface markers of B cells.

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Materials and Methods

Peripheral blood lymphocytes from 18 patients with chronic lymphocytic leukemia (CLL, blood lymphocyte counts ranging from 900/µl to 297 000/µl), 2 patients with hairy cell leukemia (HCL lymphocyte count 11,868/µl and 13,248/µl), 1 patient with leukemic lymphoplasmacytoid lymphoma (lymphocyte count 224,200/µl) and 2 patients with leukemic centrocytic lymphoma (lymphocyte count 14,200 µl and 20,230/µl) were studied. The diagnoses were based on clinical and cytological criteria and on histological examinations of lymph nodes according to the Kiel classification of malignant non-Hodgkin's lymphomas [13]. At the time of study 9 CLL patients were untreated 2 had previously received a combination chemotherapy with chlorambucil and prednisone, 1 patient was under therapy with chlorambucil, 2 were treated with prednisone, 3 with both chlorambucil and prednisone and 1 with cyclophosphamide and vincristine. The patients with HCL were studied 1 and 18 months after splenectomy when the hairy cells with typical tartrate resistant acid phosphatase isoenzyme activity comprised about 20 and 45% of blood leukocytes. No chemotherapy had been performed in these patients. The patient with lymphoplasmacytoid lymphoma was untreated. One patient with centrocytic lymphoma had previously received a combination chemotherapy the second patient was untreated.

Mononuclear cells were isolated from heparinized venous blood by centrifugation over a Ficoll-sodium metrizoate layer for 40 min at 400 g. The cells at the interface were carefully collected and were washed twice with phosphate-buffered saline. Purity of the cell suspensions was determined by differential counts of stained smears. The preparations contained about 95% lymphocytes. As judged by dye exclusion test using erythrosin B, viability was 95%.

Spontaneous rosette formation with sheep red blood cells, a property of T lymphocytes, and immunofluorescent staining of membrane-bound immunoglobulins, a marker for B cells, were performed as described previously [10]. 75–90% of the blood lymphocytes from 16 patients with CLL, both patients with centrocytic lymphoma and from the patient with lymphoplasmacytoid lymphoma were classified as B cells [W. AUGENER, unpubl. data, 1975] whereas more than 50% of the lymphocytes from 2 patients with CLL (after development of partial remission under therapy with prednisone) and from both patients with HCL were T cells.

For SEM the lymphocytes were fixed at room temperature with 2% glutaraldehyde in phosphate buffer (pH 7.4 370 mosm) and kept at 4 °C for at least 1 h. Then, the cells were collected on thin polycarbonate membrane filters of 0.8 µm porosity (Nucleopore: Thomas Co. Philadelphia, Pa.) by aspiration-filtration and were rinsed twice in phosphate buffer (pH 7.4). Subsequently the samples were dehydrated in a graded series of ethyl alcohol followed by amyl acetate/absolute alcohol and finally absolute amyl acetate, each for 1 h at 22 °C. After critical point drying (Polaron E 3000: Polaron Equipment Ltd., London England) in carbon dioxide [4] the cells were coated with thin layers of gold by a sputter coating technique in a vacuum apparatus (Balzers AG Liechtenstein) [19]. For examination of specimens a Cambridge Stereoscan S4-10 scanning electron microscope (Scientific and Medical Instruments Ltd., Cambridge England) was used at an accelerating voltage of 20 kV and with a 200 µm diameter illuminating aperture. Resolution of the SEM was

in the order of 150 Å. Hundreds of cells were scanned on the screen before recording micrographs at direct magnifications from ×1,000 to 20,000.

Results

Peripheral blood lymphocytes from all patients studied were generally spherical in shape. The size of the CLL cells and their number of surface projections (length up to 0.8 μm, breadth from 100 to 300 nm) varied from patient to patient. Lymphocytes from 3 cases with CLL (2 untreated, 1 previously treated with chlorambucil and prednisone) ranged in diameter from 4.2 to 5.4 μm (mean 4.8 μm). They displayed villous surfaces with homogeneous distribution of 60–140 stub- or finger-like microvilli per exposed part of the cell. Occasionally an accumulation of finger-like projections adjacent to a thrombocyte was observed (fig. 1).

In 10 CLL patients (7 untreated, 3 treated with chlorambucil and prednisone) the lymphocytes had a mean diameter of 4.4 μm (3.8–5.2 μm) and showed moderate numbers of surface digitations (20–60/visible surface fig. 2), sometimes with inhomogeneous distribution so that nonvillous areas as well as those densely covered with microvilli were observed. Cells from 3 treated patients ranged in diameter between 3.7 and 4.4 μm (average 4.0 μm). They were entirely smooth or had only few stub-like surface projections (up to 5–10/exposed part of the cell). Their general surface configuration appeared somewhat irregular (fig. 3). In one of these cases histological examination of a lymph node biopsy revealed extreme immaturity with clusters of prolymphocytes. There was little response to therapy and a tendency to progression of the disease with a survival time of 20 months. The other 2 patients are suffering from typical CLL since 4–5 years. In each of these 16 patients 80–90% of the lymphocytes showed uniform morphological features. Few cells differed in their surface architecture from the majority. Occasionally a monocyte with ridge-like profiles and ruffled membranes was seen. However, in 2 cases who had almost normal percentages of E-rosette forming cells after development of a partial remission under theapy with prednisone, SEM revealed a heterogeneous population concerning cell size as well as surface morphology. Most of the cells displayed a moderate number of surface projections.

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Materials and Methods

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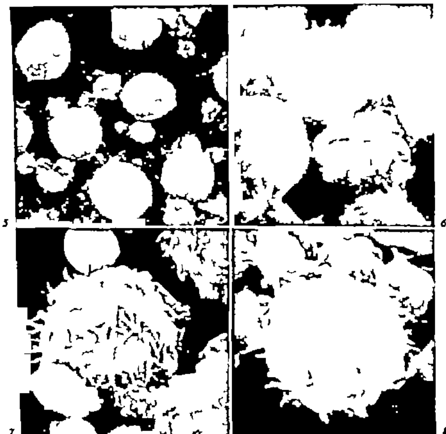


Fig 5 Leukemic centrocytic lymphoma with predominantly villous lymphocytes. × 3,750.

Fig 6. Centrocytic lymphoma, villous lymphocytes as well as 'atypical cells with minor number of long microvilli. × 7,500.

Fig 7 Hairy cell from HCL displaying numerous long thin microvilli. × 9 750.

Fig 8. Hairy cell characterized by long microvilli and ridge-like profiles as well as stub-like surface projections. × 11,250

tic lymphoma displayed rather villous cell surfaces (more than 60 microvilli/visible cell part) with a mean diameter of about 4.6 μm (fig. 5) However apart from typical villous lymphocytes we found in another case spherical cells (mean diameter about 4 0 μm) with a minor number of

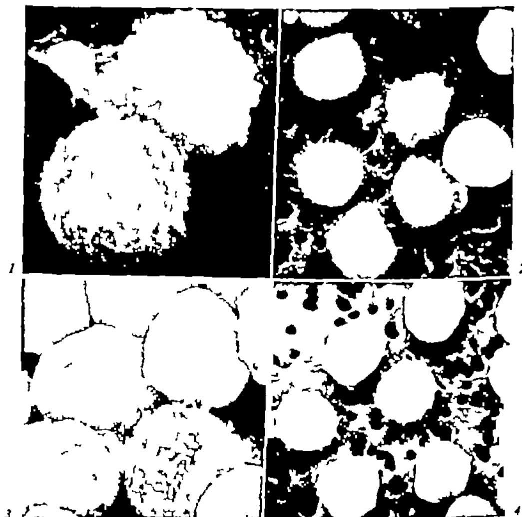


Fig 1 Villous lymphocytes from a patient with CLL. One cell shows an accumulation of microvilli adjacent to a thrombocyte × 7,500.

Fig 2 Leukemic lymphocytes from CLL with a moderate number of surface projections. On the right a normal mature erythrocyte. × 3,750

Fig 3 *Smooth lymphocytes from CLL with some irregularity of surface configuration.* × 7,500

Fig 4 Smooth lymphocytes from a case of leukemic lymphoplasmacytoid lymphoma. × 3,750

smooth surface (up to 5–10 stub-like projections/visible surface) with some degree of irregularity (fig. 4) Cell size ranged from 4 0 to 4 6 μm (mean 4.2 μm) in diameter Very few spherical lymphocytes with a more villous surface were seen Lymphocytes from both patients with centrocy

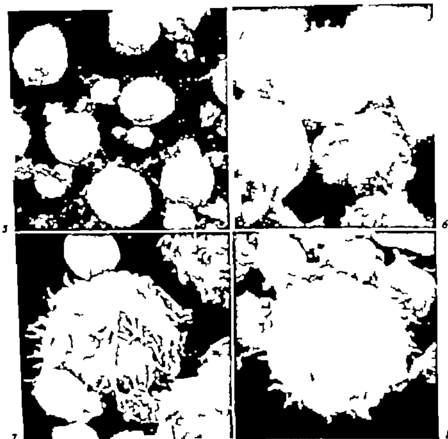


Fig 5 Leukemic centrocytic lymphoma with predominantly villous lymphocytes. × 3,750.

Fig 6. Centrocytic lymphoma: villous lymphocytes as well as 'atypical cells' with minor number of long microvilli. × 7,500.

Fig 7 Hairy cell from HCL displaying numerous long thin microvilli. × 9 750.

Fig 8 Hairy cell characterized by long microvilli and ridge-like profiles as well as stub-like surface projections. 11,250.

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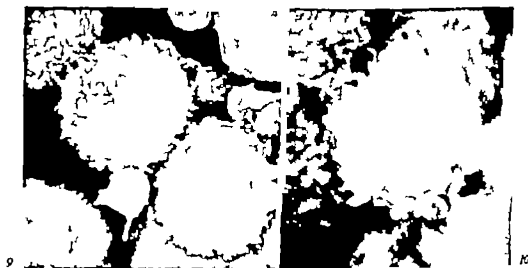


Fig 9 Hairy cell leukemia large number of very villous lymphocytes differing from typical hairy cells. × 7,500.

Fig 10 Hairy cells with ruffles and some stub-like microvilli surrounded by villous lymphocytes. × 7,500.

long surface projections (length up to 1 0 μm) and some ridge-like profiles (fig. 6)

In the patients with HCL (fig 7 8 10) the percentage of hairy cells estimated by SEM as well as by light microscopy was about 20 and 45% at the time of investigation Surface morphology of hairy cells varied to a certain extent. They showed features of both lymphocytes and monocytes by the presence of ruffles and ridge like profiles numerous long microvilli (length up to 1.5 μm, breadth 90–170 nm) and stub-like projections (breadth 75–225 nm) The cell size was comparable to that of villous lymphoytes A relatively high proportion of the remaining lymphocytes displayed more than 80 microvilli per visible surface (fig 9)

Discussion

The restriction of membrane bound immunoglobulins on CLL lymphocytes to one heavy and light chain supports the idea that in most patients with CLL a monoclonal proliferation and accumulation of leukemic B cells occurs [1 2] This interpretation is supported by our observations

indicating that about 80–90% of the peripheral blood lymphocytes from the CLL patients display a uniform surface morphology Apart from the leukemic cells there seems to exist a residual population of normal lymphocytes with a surface architecture differing from that of the majority of cells. Thus, we found a more heterogeneous population in both cases with partial remission. Lymphocytes from most patients with B-type CLL showed villous surfaces as has also been reported by POLLIACK and DE HARVEN [27] and CATOVSKY *et al* [8] However in spite of their B cell nature lymphocytes from 3 of our treated cases with CLL were almost completely smooth. Generally villous CLL lymphocytes were larger than the smooth types which applies also for normal lymphocytes [25] The diminished size and the membrane alterations of CLL lymphocytes have been described earlier and have recently been reviewed [6] Concerning absolute diameter of lymphocytes, cell shrinkage resulting from critical point drying procedures has to be considered [4 5]

Our findings suggest that cells from cases with leukemic centrocytic lymphoma have predominantly villous surfaces. However the morphological features were less homogeneous than in CLL. An almost uniform population of smooth cells were observed in a patient with lymphoplasmacytoid lymphoma.

Hairy cells from the patients with HCL had a rather complex surface architecture. They displayed features of both lymphocytes and monocytes to a varying degree. Controversial results concerning the nature of hairy cells have recently been published suggesting either a B lymphocytic or monocytic origin (9 11 14 16, 17 20]

Our SEM study on leukemic B lymphocytes indicates that a villous cell surface is not a general morphological feature of all lymphocytes with B cell membrane markers. They may show a wide spectrum of surface topology ranging from markedly villous to completely smooth. Recently CATOVSKY *et al* [8] investigated peripheral blood lymphocytes from 6 patients with acute T lymphoblastic leukemia (ALL) Cellular surfaces were either devoid of projections or had a relatively large number of short microvilli. Furthermore, there were no morphological differences between blast cells from T ALL and non T-ALL. POLLIACK and DE HARVEN [27] observed malignant T cells of the predominantly smooth type In another case of T-ALL and in a case of thymoma. T lymphocytes from a lymph node of poorly differentiated diffuse lymphocytic lymphoma (Rappaport classification) were studied by MANN *et al.* [23] They found cells with a moderate number of microvilli. Thus, it may be concluded that leukemic T

lymphocytes also do not display any characteristic pattern of surface architecture On the basis of these results the possibility has to be considered that the surface morphology of malignant human lymphocytes does not depend merely on their B or T cell nature The extreme lability of cell surface structures has been evaluated in several previous studies. In particular cellular environment [7 9] temperature changes in cell culture [22], variations in preparative methods [3 5 29] cell cycle [24] mitogens [15] cell surface contacts [29] and cellular interaction *in vitro* [18 ?1 26 28] have been shown to influence the surface morphology Thus, comparable results may only be obtained under standardized conditions. Possibly the surface morphology of peripheral blood lymphocytes primarily reflects their functional state or – as in leukemic states – the extent of membrane abnormality Furthermore, our SEM observations raise the possibility that surface architecture may be influenced by therapeutic measures or may alter with progression of the disease since all CLL patients with predominantly smooth lymphocytes were treated at the time of investigation

Acknowledgment This work was supported by the Alfred and Cläre Pott Stiftung, Essen, and by the Ministerium für Wissenschaft und Forschung, Nordrhein-Westfalen, Germany We thank Prof Dr K. Lennert Director of the Institute of Pathology University of Kiel for the histological diagnosis, and Prof Dr H. Ludwig, Director of the Department of Gynecology and Obstetrics, University of Essen, for placing his scanning electron microscope at our disposal. The excellent technical assistance of Mrs. M Funke and Miss H Metzger is gratefully acknowledged

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Preparation, Purification and *in vitro* Properties of a Serum against Human Lymphoblastic Leukemia-Associated Antigens

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Key Words. Acute lymphoblastic leukemia · Antileukemic sera · Cytotoxicity · Leukemia antigens · Tumor-associated antigens

Abstract. A serum against human lymphoblastic leukemia cells was obtained inoculating rabbits. W studied the specificity of the serum before and after particular absorptions in various clinical conditions. The serum was cytotoxic against many cases of acute lymphoblastic leukemia, against continuously cultured Burkitt's lymphoma cells and against chronic myelogenous leukemia in blast crisis. On the contrary the serum was not cytotoxic against lymphocytes of normal donors, acute lymphoblastic leukemia in remission and other lymphoproliferative disorders.

Lymphoproliferative diseases and, particularly acute lymphoblastic leukemia (ALL) were recently studied in order to identify particular antigenic modifications in neoplastic cells. This kind of immunological study of tracing cellular properties in tumors could produce useful diagnostic, prognostic and therapeutic results.

Specific antigens in leukemias were studied by various authors using specific antisera [8, 10–12]. The greatest difficulty in this kind of study is the preparation of specific antisera it is clear that an inoculated animal produces antibodies against nonspecific and assumed associated leukemic antigens. For their inoculations some authors used animals with tolerance towards human leukocyte antigen (HLA) identical normal human cells [4] or extracts of membranes of continuous cultured cells which might have cross-reacting antigens with leukemic cells [5 7 10]

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Table II. Values of CI with anti-ALL serum in 10 normal donors and in 10 patients with various diseases

Case No.	Serum dilutions 1/8	1/32	1/256
Normal donors			
1	0.03	0.02	0.01
2	0.22	0.19	0.15
3	0.03	0.06	0.01
4	0.90	0.30	0.06
5	0.83	0.08	0.00
6	0.86	0.07	0.00
7	0.14	0.06	0.01
8	0.15	0.16	0.09
9	0.27	0.03	0.00
10	0.34	0.09	0.02
Various diseases			
11 Allergy to cow' milk	0.77	0.17	0.06
12 Asthma	0.18	0.17	0.18
13 Acute bronchopneumonia	0.59	0.08	0.06
14 Macroglobulinemia	0.88	0.06	0.06
25 Encephalomyelitis	0.89	0.33	0.20
16 Carcinoma of the breast	0.67	0.33	0.18
17 Chronic mucocutaneous candidiasis	0.96	0.30	0.16
18 T cell defect	0.64	0.34	0.33
19 T cell defect	0.20	0.12	0.04
10 CVH	0.12	0.03	0.00

Serum anti-ALL(a) (I). The serum obtained as described for serum anti-ALL(a) was absorbed with lymphoblasts of different patient with ALL in acute phase (packed cells/serum ratio 1.1).

Cytotoxicity test. The sera obtained were tested *in vitro* with cells of normal donors, patients with ALL and their relatives, patients with various diseases and with continuous cultured cells.

We used the cytotoxicity technique in presence of complement as we described in another work [13]. The titre of cytotoxic activity was determined using code samples. Dilutions were made within T.C.199. Target cells were used at final concentration of 4×10^6/ml, using rabbit normal serum as the source of complement. Incubation time of cells, antisera and complement was 40 min at 37 °C. Death cells were envisaged using uptake of trypan blue.

The results are expressed with the cytotoxic index (CI) [2] and are calculated for significant antiserum dilutions.

Table I Clinical and immunological data of the 2 patients with ALL whose cells were used for immunization

Case No	Sex	Age years	WBC/µl	Blasts %	Rosettes, %		SmIg		
					E	EAC	G	A	M
1	F	5	6,000	90	12	21	ND		
2	F	2	6,800	88	7	13	0	0	0

SmIg = Surface membrane immunoglobulins IgG IgA and IgM ND = not done or not available.

We studied the problem from two points of view (1) antiserum specificity at various dilutions, and (2) specificity of antisera following a particular absorption scheme.

Material and Methods

Serum anti ALL. Leukemic lymphoblasts used for inoculation were obtained from the peripheral blood of 2 patients with ALL in acute phase whose diagnosis had been confirmed by usual clinical and laboratory methods. Pertinent data concerning these 2 patients are reported in table I.

The cells were obtained from patients before therapy Lymphoid cells were isolated by centrifugation on Ficoll-Isopaque gradient, washed twice and suspended in T C. 199 (Wellcome, England). Cells were stored at –80 °C until inoculation. The suspension of cells was injected intravenously in a rabbit at a dosage of 2×10^7 cells. This was followed by two subsequent inoculations of 10^7 cells each performed at 14 and 21 days, respectively The two inoculations were made with subcutaneous injections in different places, and an equal volume of Freund's complete adjuvant (Difco, Detroit, Mich.) was added to the cell suspension. After a period of 7 days we performed the first bleeding, and electrophoresis proved an increase of γ-globulins. At this time we sacrificed the rabbit. The serum obtained was incubated at 57 °C for 30 min and stored at –20 °C.

Serum anti-ALL(a) We absorbed the antiserum anti-ALL with an equal volume of packed cells (red blood cells A, B, Rh positive) for 20 min at 20 °C and for 1 h at +4 °C. Then we absorbed this antiserum in the same manner with a pool of normal lymphocytes (cells/serum ratio 1.2) and with the lymphocytes of the parents whose sons had given us the lymphoblastic cells for inoculation (2×10^8 lymphocytes/ml). Finally for absorption we used the lymphocytes of the same patients during the remission phase, obtained after antileukemic therapy 2×10^8 lymphocytes isolated from peripheral blood were used for 1 ml of antiserum. The absorptions were performed until the serum no longer reacted with these cells as described in table V

Table IV. Values of CI with anti-ALL serum in 10 relatives of patients with ALL (No. 1–10), in 5 patients with ALL in CR (No. 11–15), in 6 patients with other lymphoproliferative disorders (No. 16–21) and in continuous cultured cell lines (No. 22–23)

Case No.		Serum dilutions 1/8	1/32	1/256
1	Mother of case 9 (table III)	0.45	0.12	0.05
2	Father of case 9 (table III)	0.12	0.23	0.06
3	Brother of case 9 (table III)	0.48	0 07	0.06
4	Father of case 11 (table IV)	0.25	0.20	0 04
5	Mother of case 11 (table IV)	0.35	0.16	0 14
6	Mother of case 12 (table IV)	0.22	0 00	0.00
7	Father of case 12 (table IV)	0.72	0.02	0.00
8	Mother of case 13 (table IV)	0.00	0.00	0.00
9	Mother of case 2 (table I)	0.98	0.62	0.18
10	Father of case 2 (table I)	0.97	0.54	0.00
11	ALL in CR	0.20	0.18	0 05
12	ALL in CR	0.94	0 16	0.05
13	ALL in CR	0.43	0.05	0 00
14	ALL in CR (case 2, table I)	0.99	0.81	0 08
15	ALL in CR (case 1 table I)	0.40	0.11	0 04
16	Acute myelomonocytic leukemia	0.98	0.20	0.12
17	Acute myeloblastic leukemia	0.23	0.19	0.19
18	CML	1.00	0.78	0 28
19	CML in BC	1.00	0 68	0.34
20	Chronic lymphocytic leukemia	0.97	0.00	0.00
21	Lymphoma	0.18	0.07	0 00
22	Cultured RAJI cell line	1.00	0.96	0.98
23	Cultured cell line from infectious mononucleosis	0.32	[illegible]	0 01

patients we obtained an average CI of 0 78 (range 0 64–0.92 SD 0.09). We regard subjects as positive when CI is >0 45 at 1.32 dilution and we have positive controls (table II). 0/20, and positive patients with ALL (table III): 9/11 ($p<0.0001$). χ^2 between greater CI in controls and lower in ALL positive is 16.83 ($p<0.0005$).

The serum was also tested with lymphocytes of relatives of leukemic patients, of patients with ALL in complete remission (CR), or patients with different lymphoproliferative disorders and with continuous cultured cells (table IV). At a dilution of 1.32 we observed a high CI in RAJI cells (tissue-cultured cell line from a Burkitt s lymphoma patient), in 1 case of chronic myelogenous leukemia (CML) in blast crisis (BC), in 1 case of

Table III Values of CI with anti-ALL serum, and clinical and immunological data in 11 children with ALL in acute phase without therapy

Case No	WBC/µl	Blasts %	Rosettes, %		Serum CI dilutions			Comments
			E	EAC	1/8	1/32	1/256	
1	60,000	90	4	2	1.00	0.92	0.54	
2	ND	73	10	9	0.79	0.69	0.55	
3	12,000	62	15	23	0.85	0.71	0.59	
4	2,800[1]	0[1]	59[1]	13[1]				
			9[2]	0[2]	0.97[2]	0.83[2]	0.42[2]	Diagnosis on bone marrow
5	110,000	51	33	14	0.94	0.82	0.40	
6	ND	ND	8	ND	0.92	0.79	0.39	steroid therapy
7	10,000	40	14	ND	0.88	0.64	0.30	
8	80,000	90	2	4	0.99	0.87	0.68	
9	150,000	97	80	-	0,98	0.82	0.06	T cell leukemia
10	9 800	60	10	4	0.64	0.33	0.09	
11	4,000[1]	0[1]	68[1]	8[1]	0.61[1]	0.23[1]	0.04[1]	diagnosis on bone marrow

[1] Peripheral blood
[2] Bone marrow

The techniques of E rosettes (marker of T lymphocytes), and EAC rosettes (lymphocytes with receptor for C3) and surface membrane immunoglobulins (SmIg), were performed as indicated in a recent work concerning standardization of immunological techniques [3].

Cases. Both in peripheral blood and in bone marrow lymphocytes were isolated on Ficoll Isopaque gradient, washed twice and resuspended in T.C.199

The diagnosis had been confirmed by usual clinical and laboratory methods.

Results

Table II shows the data of CI with serum anti ALL in 10 normal donors and 10 patients with different diseases, all studied as controls. At a dilution of 1.32, the serum gives an average CI of 0 15 (range 0 02–0.34 SD 0 10)

The same antiserum was tested in 11 cases of ALL in acute phase and before antileukemic therapy (table III). At the same dilution of 1 32, in 9

activity against 4 cases of ALL in acute phase, against cultured RAJI cells and against 1 case of CML in BC.

This cytotoxic capacity is considerably decreased using anti-ALL(a) (I) serum, against 4 of these same cases (table VI). In fact, we observed the loss of cytotoxic capacity in 2 cases and a marked reduction in the other 2 cases in which the value of CI was halved.

Discussion

The hypothesis we considered in this work is that in the acute phase of ALL there are specific antigens. It is possible that these antigens disappear during the hematologic remission, as we see for other cell markers that become normal in remission [1]

It is clear that, when we inoculate leukemic lymphoblasts in a rabbit, we obtain a significant antibody response against different antigens and probably against HLA antigens. We underline that our antiserum is different from anti-T serum [2] because we demonstrated a low cytotoxicity in common variable hypogammaglobulinemia (CVH), where we find a great number of circulating T lymphocytes. However the results obtained in 20 controls and 9 out of 11 cases of acute phase ALL with the anti-ALL serum, demonstrate that our antiserum, in a particular dilution, can identify most leukemic patients.

Moreover we observed a cytotoxic action in 1 case of ALL with T lymphocytes at a dilution of 1.32. In this patient the following dilution is not cytotoxic, while it is active in other types of ALL with neither B nor T cells. This observation may be significant in that other authors did not find that their antisera were active against ALL patients who showed a proliferation of T lymphocytes [6]

It is interesting to note that the capacity of the antisera of identifying leukemic-associated antigens is lost in 4 cases of ALL in remission phase probably because these antigens during CR become masked or are absent.

Considering CML we remember that some authors discovered a possible cross-reactivity between CML and ALL [4]. We think that our anti-ALL serum contains specific antibodies against acute phase-associated antigens and HLA-dependent antigens. The anti-ALL(a) serum obtained by absorptions is free of antibodies against antigens independent from the acute phase. The results of cytotoxicity of serum anti-ALL(a) in the cases of ALL, RAJI cells and CML in BC, show that this antiserum has anti-

Table V Values of CI with anti-ALL(a) serum in 10 tested subjects and in cultured RAJI cell line

Case No.		Serum dilutions 1/8	1/32	1/256
1	Mother of case 2 (table I)	0.15	0.13	0.05
2	Father of case 2 (table I)	0.24	0.12	0.07
3	ALL in CR (case 2, table I)	0.07	0.03	0.00
4	ALL in CR (case 1 table I)	0.01	0.00	0.00
5	CML (case 18, table IV)	0.25	0.12	0.08
6	ALL, acute phase (case 5 table III)	0.68	0.58	0.55
7	ALL, acute phase[1] (case 6, table III)	0.82	0.69	0.60
8	ALL, acute phase (case 8 table III)	0.50	0.50	0.44
9	ALL, acute phase (case 7 table III)	0.71	0.71	0.47
10	CML in BC (case 19 table IV)	0.60	0.75	0.05
11	Cultured RAJI cell line	1.00	0.96	0.61

[1] With steroid therapy

Table VI Values of CI with anti-ALL(a) (I) serum in 3 patients with ALL in acute phase and in 1 patient with CML in blast crisis

Case No.		Serum dilutions 1/8	1/32	1/256
1	ALL (case 5 table III)	0.10	0.18	0.03
2	ALL[1] (case 6, table III)	0.45	0.25	0.08
3	ALL (case 7 table III)	0.38	0.35	0.12
4	CML in BC (case 19 table IV)	0.00	0.10	0.08

[1] With steroid therapy

ALL in remission (case 14) and in her parents (cases 9 and 10). This patient had been a donor of lymphoblasts for inoculation during her acute phase as was the patient No. 15 who had however a low CI.

In table V we report the results obtained with serum anti-ALL(a) This serum was conveniently absorbed and the effectiveness of this method is supported by the loss of cytotoxicity against lymphocytes of case 14 and of her parents. On the contrary the antiserum still showed cytotoxic

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bodies against antigens present on these cells. In fact, a successive absorption with acute phase cells [serum anti ALL(a) (I)] reduces serological activity as we described. We underline that cross reactivity between RAJI cells and leukemic cases was demonstrated by many authors [5 7 10] Furthermore, cross reactivity between anti ALL sera and CML in BC has been reported [6]

It is obvious that a specific anti ALL serum has a great importance in general studies on leukemic diseases and lymphoproliferative disorders. But the problem is surely more complicated, because we could find aspecific mitotic antigens in many pathological conditions [9] or because leukemic cells can be more fragile than normal cells.

We would like to obtain more information about the nature of cytotoxicity in presence of complement, because we think that a standard use of this method of screening can expedite the diagnosis of some types of leukemic diseases.

Acknowledgments. We would like to thank Dr D ROBATI (Hospital B. Gesù, Rome) Dr G RAGONA, D R. D AMELIO, Dr C. MASTROIANNI and Dr J FIGUEROA (Department of Clinical Medicine III University of Rome) for their constructive assistance.

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other group of similar patients who were not given heparin served as controls.

Patients

31 patients (group A) undergoing major operation, who received subcutaneously 5,000 IU of heparin the evening before operation and every 12 h thereafter for at least 10 days, were studied. Ten patients were males and 21 females. The mean age was 53 years (range 24–76 years).

Another group of 15 patients (group B) undergoing similarly major operations, without heparin, served as controls. In this group 6 patients were males and 9 females. The mean age was 54 years (range 23–75 years).

Patients with bleeding tendency or malignant disease were excluded from the study. All patients did not receive drugs affecting platelet function for at least 15 days before operation and during the study.

Methods

Blood was anticoagulated with trisodium citrate (3.2%). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by differential centrifugation [19].

The following platelet tests were applied: (1) Platelet count [3]. (2) The template bleeding time [13]. (3) The SALZMAN [20] test of platelet retention in glass bead column (adhesiveness) slightly modified [18]. (4) Platelet aggregation was tested by the turbidometric technique [14] using collagen and ADP (1 μM) as aggregating agents [19]. (5) ADP release from platelets by collagen was assessed as described elsewhere [19].

The degree of aggregation was expressed as percentage of maximal fall in optical density. In the collagen-induced aggregation the delay period and the slope of the aggregation curve were also estimated. All tests were performed the day before operation (before heparin administration) and on the 4th postoperative day. Platelet count was performed additionally on the 2nd, 3rd and 6th postoperative days.

To compaire the differences of the mean values within each group and between the groups the paired t test and the t test for small samples were used, respectively.

Results

Platelet count. In the controls the platelet count was significantly decreased ($p<0.05$) on the 2nd postoperative (PO) day ($M = 260$, $SD = 51.7$ platelets $\times 10^9$) compared to the preoperative levels ($M = 278.5$

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Postoperative Platelet Function in Patients on Small Subcutaneous Doses of Heparin

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Key Words Blood coagulation Heparin Platelet function Postoperative platelet changes Thrombosis

Abstract Several platelet function tests were performed on 31 patients undergoing major operations, who received prophylactically small subcutaneous doses of heparin. A group of 15 similar patients without heparin served as controls. It was found that postoperatively in both groups (a) the platelet retention in glass bead column was significantly increased ($p<0.001$), (b) the platelet aggregation by ADP 1 μM was slightly increased, (c) the collagen-induced aggregation, ADP release and the bleeding time remained unchanged and (d) the platelet counts decreased in the first 2 postoperative days and increased thereafter There was no difference between patients on heparin and controls.

The role of platelets in postoperative deep venous thrombosis is not clear The mean platelet count decreases during the first postoperative day [4 7] but thereafter increases [1 9] The retention of platelets in glass bead column, platelet aggregation by ADP and electrophoretic mobility of platelets by ADP increase postoperatively [1 6, 9 10 12] How ever it is not defined whether these changes are the result of platelet 'hy peractivity *leading to thrombosis or are secondary to operation.*

It has been recently found that small subcutaneous doses of heparin given before and after surgery reduce the incidence of postoperative deep vein thrombosis [8 11] However little is known of postoperative platelet changes. If platelets are related to formation of postoperative deep vein thrombosis it is reasonable to expect reduction of postoperative platelet changes after heparin administration

We investigated platelet number and function in patients undergoing major operation who received small subcutaneous doses of heparin An

Table II. Template bleeding time and ADP release in both groups preoperatively and postoperatively

Group	Template bleeding time, min				ADP release, µg/3 10⁸ platelets			
	preoperatively		postoperatively		preoperatively		postoperatively	
	M	SD	M	SD	M	SD	M	SD
A	4.7	1.2	4.6	1.5	5.8	4.3	6.2	4.8
B	4.3	1.2	4.1	1.1	5.7	3.3	5.7	4.3

M = Mean value SD = standard deviation.

Discussion

Experimental studies of the *in vitro* and *in vivo* effect of heparin on platelet number and function gave controversial results [2, 5 17 21, 22] ZUCKER [23] in a recent review article, concluded that 'heparin enhances primary aggregation, induces aggregation by causing the release reaction, and as a result, may reduce the release reaction subsequently provoked by other stimuli such as epinephrine In preliminary experiments on healthy subjects we did not find changes of platelet number and function 2 and 12 h after subcutaneous administration of 5,000 IU of heparin [unpubl. observations]

In this study the platelet function test showed similar changes post operatively in both groups of patients. The changes consisted of a significant increase of the retention of platelets in glass bead column and a slight increase of the ADP-induced aggregation (at final concentration 1 µM). These findings are at variance with those of O'BRIEN *et al.* [15, 16] who also studied patients undergoing operation and observed that the ADP-induced aggregation was decreased in the controls, whilst it was normal in patients receiving small subcutaneous doses of heparin. However these authors tested their patients immediately after surgery and not on the 4th postoperative day as in the present experiments. It seems that our findings do not support the hypothesis that platelets are an important factor in the production of the postoperative deep vein thrombosis.

On the other hand, this study showed difference of changes of platelet count postoperatively between the heparinized patients and the controls. In both groups of patients the platelet count decreased on the 2nd post

Table I Collagen-induced aggregation in group A and group B preoperatively and postoperatively

Group	Preoperatively						Postoperatively					
	delayed period sec		slope 0		total aggregation %		delayed period sec		slope 0		total aggregation %	
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
A	26.3	10.8	82.4	4.4	85 0	8.5	26.3	6.8	80.7	3.6	83 4	7 6
B	24 0	7.8	83 4	2.8	88 0	6.4	24.2	6.4	82.6	2.8	84.7	6.3

M = Mean value SD = standard deviation.

SD = 47.5 platelets ×10³), restored to the preoperative levels on the 3rd PO day (M = 275 SD = 51.3 platelets ×10³) and followed by a significant increase ($p<0\ 05$) on the 4th and 6th PO days (4th PO day M = 301 SD = 58 6 platelets ×10³ 6th PO day M = 308 SD = 50 6 platelets ×10³) In the group of patients on heparin the platelet count also showed a significant decrease ($p<0\ 01$) on the 2nd PO day (preoperatively M = 266.5 SD = 52 platelets ×10³ 2nd PO day M = 251 SD = 57 platelets ×10³) but it remained low on the 3rd PO day (M = 252.8, SD = 56 platelets ×10³) and restored to the preoperative levels on the 4th PO day (M = 267.9 SD = 56 platelets ×10³) A slight but not significant increase was noted on the 6th PO day (M = 283 SD = 60 platelets × 10³)

Platelet retention in glass bead column Platelet adhesiveness was significantly increased ($p<0\ 001$) postoperatively in both groups (group A preoperatively M = 47 8, SD = 7 6%, postoperatively M = 56 0 SD = 9 1% group B preoperatively M = 48.3 SD = 8 0%, postoperatively M = 58 7 SD = 7 1%)

The ADP-induced aggregation at final concentration 1 μM was slightly increased postoperatively in both groups (group A preoperatively M = 28.5 SD = 8 7%, postoperatively 32.2, SD = 10.9% group B preoperatively 28.4 SD = 10.58%, postoperatively 31.5 SD = 8 6%). This increase was not statistically significant.

The collagen-induced aggregation (table I), ADP release by collagen (table II) and the template bleeding time (table II) remained unchanged postoperatively in both groups.

Table II. Template bleeding time and ADP release in both groups preoperatively and postoperatively

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Platelet retention in glass bead column. Platelet adhesiveness was significantly increased ($p<0.001$) postoperatively in both groups (group A preoperatively M = 47.8 SD = 7.6%, postoperatively M = 56.0 SD = 9.1% group B preoperatively M = 48.3 SD = 8.0%, postoperatively M = 58.7 SD = 7.1%).

The ADP-induced aggregation at final concentration 1 μM was slightly increased postoperatively in both groups (group A. preoperatively M = 28.5 SD = 8.7%, postoperatively 32.2, SD = 10.9% group B preoperatively 28.4 SD = 10.58%, postoperatively 31.5 SD = 8.6%) This increase was not statistically significant.

The collagen-induced aggregation (table I) ADP release by collagen (table II) and the template bleeding time (table II) remained unchanged postoperatively in both groups.

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operative day but thereafter in the heparinized patients the platelet number did not promptly rise to the preoperative level and did not show significant increment on the 6th postoperative day as did the controls.

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Table 1 Information about the patients

Patient No.	Age	Sex	Diagnosis	Histological subclassification	Clinical stage	Previous therapy[a]	Duration of the disease prior to DTIC months	Number of DTIC courses	Response[a]	Duration of remission weeks
1	44	F	HD	LD	IV B	MOPP VIMeP	13	1	–	–
2	31	M	HD	MC	III B	MOPP	22	3	CR	36[a]
3	44	F	HD	NS	IV B	MOPP VIMeP R Bleo	56	2	PR	9
4	32	M	HD	NS	IV B	MOPP	28	2	PR	13
5	35	M	HD	not determined	IV B	MOPP	30	3	PR	16[a]
6	49	M	HD	LP	III B	MOPP	25	2	CR	10[a]
7	26	F	HD	not determined	IV B	MOPP VM-26	32	3	PR	18
8	30	F	HD	MC	IV B	MOPP	12	2	PR	8
9	29	M	HD	MC	IV B	MOPP	15	3	PR	9
10	25	F	HD	NS	III B	MOPP	12	2	PR	16
11	27	M	RCS		IV B	COP Pepti-chemio	26	2	PR	3
12	52	F	RCS	not determined	IV B	COP	11	2	F	
13	43	M	RCS	not determined	IV B		2	2	PR	4
14	55	M	RCS	not determined	IV B	COP	24	2	PR	6
15	44	M	LS	not determined	IV B	COP Leukeran R	22	2	F	

HD = Hodgkin's disease · RCS = reticulum cell sarcoma · LS = lymphosarcoma.

LP = Lymphocytic predominance · NS = nodular sclerosing · MC = mixed cellularity · LD = lymphocyte-depletion.

MOPP = Mustard, oncovin, procarbazine, prednisone · COP = cyclophosphamide, oncovine, prednisone · ViMeP = vinblastine, methotrexate, prednisone · Bleo = bleomycine · R = Radiotherapy

F = Failure · PR = partial remission · CR = complete remission.

Remission not yet terminated.

Acta haemat. 57 272–278 (1977)

Imidazole Carboxamide (DTIC) in the Treatment of Advanced Lymphomas

Efficacy of DTIC in Cases which Fail to Respond to Conventional Chemotherapeutic Combinations

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Key Words. Chemotherapy · Lymphomas · Hodgkin's disease · Imidazole carboxamide (DTIC) · Malignant lymphomas

Abstract. 15 patients with malignant lymphomas (stage III B or IV) who had become resistant to previous combination chemotherapy were treated with DTIC. The drug was administered intravenously as a single agent in doses of 300 mg/m² on 5 consecutive days, once a month. The results demonstrate good responses in Hodgkin's disease, while in non-Hodgkin's lymphomas only incomplete and short remissions or failures were recorded. The only untoward side effects were nausea, vomiting and pain in the vein during the injection.

DTIC [5-(3,3-dimethyl 1 triazeno)-imidazole-4-carboxamide, NSC 45388] marketed recently under the name DTIC – Dome (Dome Laboratories) is a potent antitumour drug, particularly successful in the treatment of metastatic malignant melanoma [2, 9, 10, 15, 19]. Numerous reports indicate that it can produce a remission of over 20%. Less frequently DTIC has been used in the treatment of variety of human cancers, either alone [7, 8, 16] or in combinations with other cytotoxic drugs [6, 11, 18]. This report will document our experience in determining the activity of DTIC treatment in patients with generalized forms of malignant lymphomas, who fail to respond to conventional chemotherapeutic combinations.

[1] The authors wish to thank Dr. M. SLAVIK (chief Investigational Drug Branch, National Cancer Institute, Bethesda) for kind supply of DTIC.

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3	44	F	HD	NS	IV B	MOPP V MeP R Bleo	56	2	PR	9
4	32	M	HD	NS	IV B	MOPP	28	2	PR	13
5	35	M	HD	not determined	IV B	MOPP	30	3	PR	16[a]
6	49	M	HD	LP	III B	MOPP	25	2	CR	10[a]
7	26	F	HD	not determined	IV B	MOPP VM-26	32	3	PR	18
8	30	F	HD	MC	IV B	MOPP	12	2	PR	8
9	29	M	HD	MC	IV B	MOPP	15	3	PR	9
10	25	F	HD	NS	III B	MOPP	12	2	PR	16
11	27	M	RCS		IV B	COP Papci-chemio	26	2	PR	3
12	52	F	RCS	not determined	IV B	COP	11	2	F	
13	43	M	RCS		IV B			2	PR	4
14	55	M	RCS		IV B	COP	24	2	PR	6
15	44	M	LS		IV B	COP Leukeran R	22	2	F	

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LP = Lymphocytic predominance · NS = nodular sclerosing · MC = mixed cellularity · LD = lymphocyte-depletion.

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Key Words. Chemotherapy Lymphomas Hodgkin's disease Imidazole carboxamide (DTIC) Malignant lymphomas

Abstract. 15 patients with malignant lymphomas (stage III B or IV) who had become resistant to previous combination chemotherapy were treated with DTIC. The drug was administered intravenously as a single agent in doses of 300 mg/m² on 5 consecutive days, once a month. The results demonstrate good responses in Hodgkin's disease, while in non-Hodgkin's lymphomas only incomplete and short remissions or failures were recorded. The only untoward side effects were nausea, vomiting and pain in the vein during the injection.

DTIC [5-(3,3-dimethyl 1 triazeno)-imidazole-4-carboxamide, NSC 45388] marketed recently under the name DTIC – Dome (Dome Laboratories) is a potent antitumour drug, particularly successful in the treatment of metastatic malignant melanoma [2, 9 10 15 19] Numerous reports indicate that it can produce a remission of over 20%. Less frequently DTIC has been used in the treatment of variety of human cancers, either alone [7 8, 16] or in combinations with other cytotoxic drugs [6 11 18] This report will document our experience in determining the activity of DTIC treatment in patients with generalized forms of malignant lymphomas, who fail to respond to conventional chemotherapeutic combinations.

The authors wish to thank Dr M. Slavik (chief, Investigational Drug Branch, National Cancer Institute, Bethesda) for kind supply of DTIC.

Table 1 Information about the patients

Patient No.	Age	Sex	Diagnosis	Histological subclassification	Clinical stage	Previous therapy[a]	Duration of the disease prior to DTIC months	Number of DTIC courses	Response[b]	Duration of remission weeks
1	44	F	HD	LD	IV B	MOPP VIMeP	13	1		
2	31	M	HD	MC	III B	MOPP	22	3	CR	36[c]
3	44	F	HD	NS	IV B	MOPP VIMeP R Bleo	56	2	PR	9
4	32	M	HD	NS	IV B	MOPP	28	2	PR	13
5	35	M	HD	not determined	IV B	MOPP	30	3	PR	16[c]
6	49	M	HD	LP	III B	MOPP	25	2	CR	10[c]
7	26	F	HD	not determined	IV B	MOPP VM 26	32	3	PR	18
8	30	F	HD	MC	IV B	MOPP	12	2	PR	8
9	29	M	HD	MC	IV B	MOPP	15	3	PR	9
10	25	F	HD	NS	III B	MOPP	12	2	PR	16
11	27	M	RCS		IV B	COP Pepti-chemio	26	2	PR	3
12	52	F	RCS	not determined	IV B	COP	11	2	F	
13	43	M	RCS		IV B		2	2	PR	4
14	55	M	RCS		IV B	COP	24	2	PR	6
15	44	M	LS		IV B	COP Leukeran R	22	2	F	

HD = Hodgkin disease RCS = reticulum cell sarcoma LS = lymphosarcoma.

LP = Lymphocytic predominance NS = nodular sclerosing; MC = mixed cellularity LD = lymphocyte depletion.

MOPP = Mustard, oncovin, procarbazine, prednisone COP = cyclophosphamide, oncovine, prednisone VIMeP = vinblastine, methotrexate, prednisone Bleo = bleomycine R = Radiotherapy

F = Failure PR = partial remission CR = complete remission.

Remission not yet terminated.

Table II Clinical features of treated patients and influence of DTIC therapy

Disease	Patient No	Subj complaints	Fever	ESR	Splenomegaly	Hepatomegaly	Adenomegaly peripheral	Adenomegaly mediastinal	Adenomegaly abdominal	Organ involvement
HD	1	0	0	0	0	0	0	0	0	
	2	c	c	c	c	–	c	c	–	–
	3	p	c	p	p	p	p	p	–	p (L, P)
	4	c	c	p	p	–	p	p	–	–
	5	c	c	c	–	c	c	p	–	–
	6	c	c	c	c	–	c	c	–	
	7	c	c	p	c	c	c	p	p	–
	8	c	c	p	–	c	p	p	–	0 (L)
	9	c	c	c	c	p	c	p	–	–
	10	c	–	p	c	–	p	p	p	–
RCS	11	c	c	p	S	p	p	p	0	–
	12	p	0	0	0	0	0	–	–	0 (C)
	13	c	c	p	p	–	p	–	–	p (M)
	14	p	c	p	p	–	p	–	–	–
LS	15	p	p	0	S	0	0	0	–	0 (M)

HD = Hodgkin s disease RCS = reticulum cell sarcoma LS = lymphosarcoma.
ESR = Erythrocyte sedimentation rate 0 = no response p = partial regression c = complete regression S = patient after splenectomy
L = lungs P = pericard C = colon M = bone marrow

Patients and Methods

15 patients with malignant lymphoma (stage III B or IV) were eligible for the DTIC therapy all of whom had received previous therapy Ten, patients with Hodgkin s disease had been previously treated with DE VITA's MOPP cyclic regimen [5] as modified by KLENER and DONNER [13] 5 patients with non-Hodgkin s lymphoma had received COP combination [12] The diagnosis had been established from biopsy of lymph node by the usual criteria, before the first treatment was started. All patients were put on DTIC treatment when they relapsed or had become resistant to previous therapy DTIC was administered in doses of 300 mg/m /day Five day course was repeated at 1-month intervals. All patients but one received at least two treatment courses. If the disease was progressive the study was discontinued and the patient was put on another drug regimen. Prior to initiation of DTIC treatment, complete physical and laboratory examinations were performed, including peripher

al haemogram, erythrocyte sedimentation rate, liver function tests, chest roentgenograms and other examinations if needed (skeletal radiography abdominal lymphangiography bone marrow etc.). Patients were regularly followed up once fortnight. Response to treatment was evaluated according to criteria described by CARBONE and SETTIN [1].

Results

12 patients (80%) responded to the treatment (table I) However only 2 patients among them achieved complete remission (13.3 %), while the others had objective, but incomplete response. Detailed informations about the patients are summarized in table I. Two patients did not have clear objective response after the first course of therapy but responded to subsequent course. Subjective improvement was noted in all but 1 patient (No. 1) with far advanced Hodgkin s disease. Objective response was expressed in the majority of patients by immediate decrease of fever decrease in erythrocyte sedimentation rate, while palpable lymph nodes, liver or spleen diminished within several weeks after the onset of the treatment. Antitumour effect was observed even in cases with far advanced disease which failed to respond to different modalities of prior chemotherapy The clinical features of treated patients and the influence of DTIC therapy are summarized in table II.

DTIC treatment was well tolerated. The most frequent side effects observed were nausea (14 out of 15 patients) and vomiting (11 patients). The symptoms were most severe after the first injection of the initial course. Ten patients complained of pain in the vein during rapid intravenous push. However thrombosis was never observed. No haematological toxicity was recorded, though in some patients, treatment was started with leucocyte or platelet counts under lower limits of the normal values. A moderate decrease of leucocyte counts was sometimes observed within 2 weeks after the treatment, but in no case life-threatening leucopenia was recorded. Platelet counts, if decreased after therapy recovered spontaneously within a short period. No bleeding manifestation in relation to the therapy was observed. Liver kidney or bone marrow toxicity was not found.

Discussion

Numerous reports have been published reporting good results of combination chemotherapy in advanced Hodgkin s disease or non-Hodgkin s lymphomas [5 12, 13] However treatment of relapses still remains a se-

rious problem It has been suggested to add further components to the chemotherapeutic combination [3] but leucopenia and/or thrombocytopenia often prevent such a major chemotherapy On the other hand, single agent therapy is usually unsuccessful [4] That is why new approaches are still looked for In the present study we demonstrated that DTIC is a valuable drug in such situations High efficacy of the drug might perhaps be explained by its mechanism of action, as DTIC has been proved to have alkylating as well as antimetabolic activity [10] Furthermore, SAUNDERS and SCHULTZ [17] demonstrated interaction of DTIC with SH groups, which also may play a part in its antitumour action. It could be, of course, contended that 13% of complete remissions in our series of patients is not convincing, but it should be kept in mind that all patients had been treated previously and were unresponsive to prior chemotherapy From this point of view 80% of all responders, though most of them achieved only incomplete remission is evaluable as a successful treatment. The great advantage of DTIC is its low haematological toxicity No serious leucopenia and/or thrombocytopenia were recorded Furthermore, *in vitro* studies indicate that DTIC did not influence platelet functions, as many other cytotoxic drugs do [14] Gastrointestinal toxicity was rather high, but rapid onset of tolerance was noted. In many cases, thiethylperazine (Torecan® Sandoz) can reduce substantially these untoward side effects. Although the majority of patients complained severe pain in vein during intravenous administration venous thrombosis or obliteration in consequence of DTIC therapy was never observed. Nevertheless, it should perhaps be advisible to inject the drug in a more diluted solution

In conclusion, we state that DTIC is a useful agent for the treatment of advanced lymphomas, particularly for its effectiveness even in previously treated cases. Lower response rate in non-Hodgkin s lymphomas may be connected with their general lower sensitivity to chemotherapy but this assumption cannot be maintained owing to the rather limited number of cases in our study but one is certain that further study using different treatment schedules, or combining DTIC with other cytotoxic drugs might prove to be even more efficacious.

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Large Granules and Lysosomal Fusion in Human Chediak Higashi White Blood Cells

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Key Words. Acid phosphatase   Chediak Higashi syndrome   Cytochemistry   Electron microscopy   Lysosomes   Peroxidase

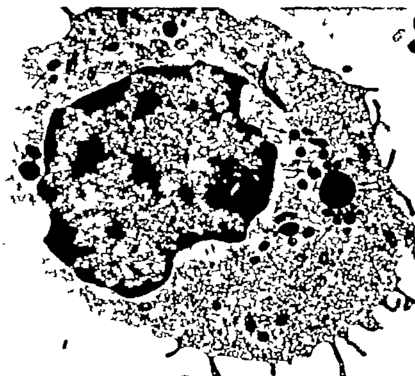
Abstract. The phenomenon of giant anomalous lysosome formation in human Chediak-Higashi syndrome leukocytes was analyzed. Ultrastructure findings combined with cytochemical procedures for visualizing acid phosphatase and peroxidase activity showed giant anomalous granules in addition to normal, small and enlarged granules. Massive granules in lymphocytes had an appearance and structure different from those found in other leukocytes. The giant granules seem to be a product of an active fusion between primary and secondary normal sized or enlarged lysosomes. This fusion occurs in polymorphonuclear neutrophils, eosinophils and in monocytes. No fusion was found in lymphocyte granules.

The Chediak-Higashi syndrome (CH) is a rare disorder determined by one autosomal recessive gene and is lethal in the homozygous state [1]. This disorder occurs in man [2, 3] and animals [4–7]. It is clinically manifested by pancytopenia, lymphoadenopathy, hepatosplenomegaly, photophobia, partial albinism in most cases, and increased susceptibility to infection. Studies of leukocyte function have documented defective granulocyte chemotaxis [8, 9] and impaired intracellular destruction of phagocytized bacteria [10] as well as reduced quantities and abnormal distribution of lysosomal enzymes [11, 12].

Cytologically, the prominent characteristic findings are large anomalous granules in granule-containing cells [13–15]. However, leukocyte granule abnormalities are pathognomonic for this syndrome [1–3, 6, 16]. The presence of acid phosphatase and peroxidase activity determined by cytochemistry techniques led to the conclusion that these large granules repre-

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F 1 Electron micrograph of immature granulocyte from peripheral blood of patient with the CH syndrome. Large number of normal-sized granules distributed in the cytoplasm (arrow) and surrounding large granule (double arrow) characteristic of this syndrome. Numerous mitochondria (M) can also be seen. N = Nucleus × 12,000.

Results

Morphological observations In peripheral blood, MGG staining and phase microscopy revealed anomalous granules in granulocytes, monocytes and lymphocytes. In the bone marrow granulocyte precursors showed giant vacuoles in the cytoplasm as well as the typical inclusion bodies.

Electron microscopy Granulocytes and monocytes in CH blood cells showed, in addition to normal small granules, enlarged and giant ones

sent lysosome varieties [1 17] Furthermore, studies in CH cells have indicated a disturbance in their lipid metabolism [18 19] It has been suggested, on the basis of ultrastructure of white blood cells of man and animals that the formation of abnormal granules may be due to a process of lysosomal fusion [17 20] In CH beige mouse hepatocytes, the giant lysosomes are formed from the Golgi endoplasmic reticulum lysosome (GERL) elements [21]

The present investigation is a study of the structure and the formation, through a process of fusion, of the anomalous granules in human CH leukocytes. The identification of the fusing lysosomes, as well as the formation of the giant granules, was based on their enzymatic staining and structural analogies.

Materials and Methods

Peripheral blood buffy coat and bone marrow from a 4-year-old non-albino male were examined

Morphologic studies Using the May-Grünwald-Giemsa stain (MGG), morphologic studies were performed on smears of peripheral blood, micro buffy coat and bone marrow cells. Phase-contrast microscopy was used to examine fresh preparations.

Electron microscopy Examinations were performed on buffy coat obtained from heparinized (200 IU/10 ml) peripheral blood and bone marrow cells, fixed in 2.5% glutaraldehyde in 0.2 M Na-cacodylate-HCl buffer (pH 7.2) containing 7% sucrose and kept at 4 C for 2 h. The samples were washed with Na-cacodylate, postfixed in 1% OsO_4 in cacodylate HCl buffer dehydrated in graded ethanols followed by propylene oxide and embedded in Epon 812 as described by LUFT [22] Sections were cut with LKB ultratome, stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

Ultrastructural cytochemistry Acid phosphatase activity was demonstrated as follows: peripheral blood and bone marrow cells were fixed in 1 / glutaraldehyde in 0.2 M Na-cacodylate-HCl buffer (pH 7.2) for 15 min at 4 °C. The cells were then washed 3 times in the same cacodylate buffer The fixed cells were suspended in Gomori incubation media [23] and maintained at 37 C, pH 4.8, for 2 h. Following incubation, the cells were washed three times in Na-cacodylate buffer and prepared for electron microscopy as described above. Staining with uranyl acetate and lead citrate was performed fter testing the presence of the enzyme activity product on unstained sections. As a control for the specificity of the staining method, fixed cells were incubated with the incubation solution lacking the substrate β-glycerophosphate.

For demonstration of peroxidase activity the samples were incubated in Gram-Karnovsky medium containing 3,3 diaminobenzidine (DAB) as substrate for 15 min [24]. Following incubation, the samples were treated as described above for acid phosphatase activity



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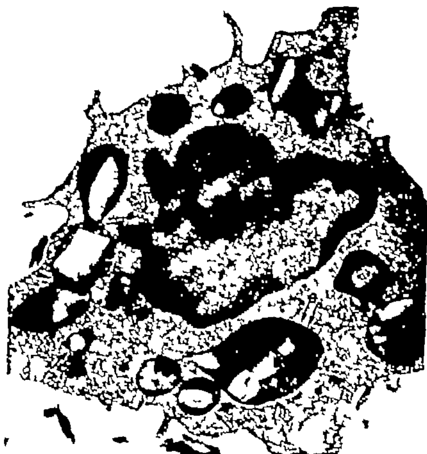


Fig 2 Eosinophilic polymorphonuclear distinguished by its large size granules and the presence of a crystalloid in most of them (arrows) An extremely large granule (double arrows) containing multiple crystalloids makes it seem likely that these giant granules are formed by fusion. Fusion between two, and between three eosinophilic granules are seen (arrowheads). × 12,000

(fig. 1–3) Scanning of many cells indicates that the latter are a product of an active fusion process of primary and secondary lysosomes. Giant anomalous granules were present in various stages of cell maturation but appeared predominantly in young myeloid cells. The lysosomal nature of these granules was evidenced by their content of characteristic lysosomal enzymes, as manifested by ultracytochemical positive staining for peroxidase and acid phosphatase reaction (fig 5 6) Most neutrophils and monocytes contained the anomalous lysosomes presenting one or more giant granules surrounded by some others of normal size (fig 1–3) Beside their

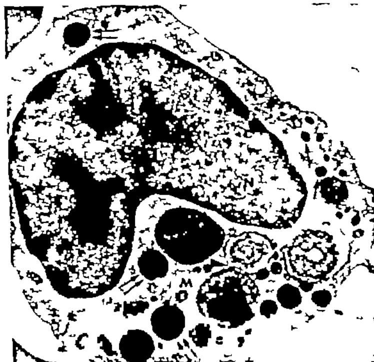


Fig 3 Neutrophilic metamyelocyte showing common small lysosomes (arrows), large granules (double arrows) and giant fusing lysosomes (arrowhead). N = Nucleus M = mitochondria. 18,000.

different size, the granules were pleomorphic in nature and contained uniform or amorphous materials.

Some of the giant lysosomes contained electron-lucent vacuoles which were interpreted as being lipid droplets, probably washed out during the preparation processes (fig. 6). Vacuolization in the cytoplasm of granulocytes was a common finding and many of the granulocytes had large destroyed areas which showed acid phosphatase and peroxidase activities (Fig. 5) Eosinophil granules were also enlarged with various configurations, indicating an active fusion and containing one or more crystaloids of unusual size and shape (fig).

Variation in the number and configuration of the fusing lysosomes was found fusion was observed between lysosomes of the same or with differ

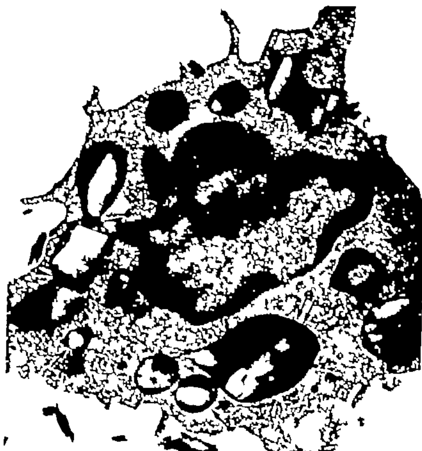


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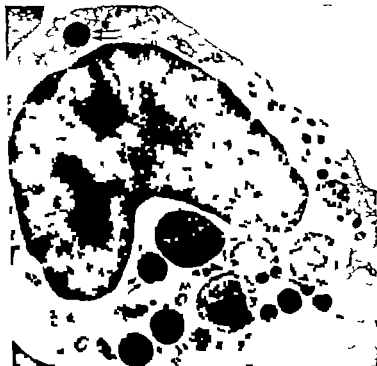


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Fig 4 Tubular structures in a small clear lymphocyte. Kidney-shaped nucleus, condensation of chromatin along the nuclear envelope. Few mitochondria (M), ribosomes and a giant organelle (arrowheads) containing tubule filled structures (in set) The tubular elements are sectioned longitudinally and transversely × 13,000. *Inset* × 40,000.

ent sizes and shapes between primary or secondary lysosomes, as well as between granules in different stages of lysosomal activity (fig. 3) The lipid laden lysosomes were observed fusing with one or more granules forming a complex of giant granules (fig 6)

On the other hand, no fusion was found in lymphocytes. Anomalous large granules in circulating lymphocytes showed cytoplasmic inclusions containing a mass of microtubular-like structures having two types of different diameters and arrangements One type contained few microtubules, 300–350 A in diameter scattered in the granules. The second type consisted of a large mass of microtubules, 150–200 A in diameter packed

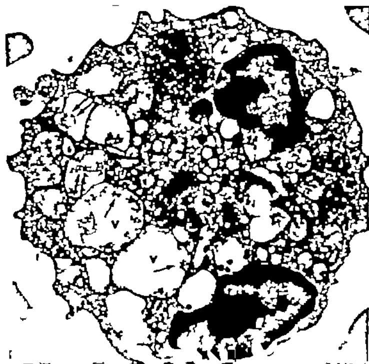


Fig. 5 Neutrophil polymorphonuclear from peripheral blood containing characteristic cytoplasmic inclusions stained by enzymatic reaction product. The sites of acid phosphatase activity are indicated by deposits of lead phosphate. The cytoplasm is filled with numerous vacuoles (secondary lysosomes). Note enzymatic reaction in giant lysosome (arrowhead) and acid phosphatase reaction product in wall area (arrows) of the vacuoles (V), and within the cytoplasmic granules and containing heterogenous material (double arrows). 90,000

tightly together and occupying most of the inclusion space (fig. 4). The diameter of the microtubules in each inclusion was uniform. The different types of inclusions could be found located in the same cell.

Discussion

The lysosomal nature of the enlarged and giant fusing granules present in leukocytes in CH was revealed by the cytochemical reactions indicating



Fig 6 Electron micrograph of an immature granulocyte from bone marrow stained by peroxidase reaction product, showing various stages of the fusing lysosomes. The peroxidase staining appears as an amorphous precipitate localized in anomalous lysosomes (arrows) Point of connection and fusion between lysosomes. Lipid droplets are nonreactive (L). × 12,600

acid phosphatase activity in addition to the peroxidase staining. The massive granules in lymphocytes had an appearance and structure different from those observed in other white blood cells. It seems that the large granules in the lymphocytes were formed without a fusion process and appeared in their protein component to be different from those observed in other leukocytes. Single giant organelles filled with masses of tubular elements seem to appear in CH in a higher percentage of lymphocytes than those found in normal cases [25]

Naturally occurring fusion of lysosomes is of great importance in the execution of the physiological role of lysosomes and their enzymatic content. Two kinds of formation of the anomalous large granules in CH were previously suggested. The first dealt with the formation of the anomalous lysosomes in liver cells in beige mouse, and indicated that hepatocytic giant lysosomes arise from GERL elements [21]. The second suggested that the giant lysosomes are formed through a process of fusion observed in CH mink and beige mouse leukocytes [20, 26]. The present study indicates that giant granules resulting from lysosomal fusion can occur both in early or late stages of cell maturation, as well as between primary lysosomes and phagolysosomes or even between two secondary lysosomes. This finding is in contrast with the observation in the CH beige mouse leukocytes in which the giant granules were found to be the result of fusing of lysosomes having a similar structural appearance [26].

The fact that in the presence of a surfactant normal leukocytes show an increased enzymatic activity similar to that found in CH would suggest that the CH has a defective lysosomal membrane structure [27]. A deficiency in membrane stability of the cytoplasmic granules in CH was also indicated by ultrastructure studies [28]. This change in structure would result in an increased permeability and eventual osmotic rupture. The appearance of giant lysosomal vacuoles in the early myeloid cells may also be explained by a process of digestion due to release of hydrolytic enzymes. A white blood cell turnover increase due to an intramedullary destruction and an increased serum muramidase level has been reported [29].

Membrane instability and abnormal active lysosomal fusion processes may be explained by postulating the presence of surface active molecules in CH cytoplasmic granules [30]. This may be produced by a disturbed lipid metabolism, favoring the formation of globular micelles of the lipid particles, if compared with the bimolecular phospholipid leaflet configuration of the lysosomal membrane.

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Antihaemophilic Factor A Activity, F VIII Related Antigen and von Willebrand Factor in Hepatic Cirrhosis

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Key Words Antihaemophilic factor Blood coagulation F VIII-related antigen Liver cirrhosis von Willebrand factor

Abstract F VIII activity F VIII-related antigen and von Willebrand factor were measured in 46 patients with hepatic cirrhosis and in 30 normal individuals. These parameters were significantly higher in hepatic cirrhosis than in the controls. Linear relationships between F VIII activity and F VIII related antigen and between F VIII related antigen and von Willebrand factor were found in patients with hepatic cirrhosis as well as in normal individuals. However in both groups no relationship between F VIII activity and von Willebrand factor was present. The existence of a low-grade intravascular coagulation in hepatic cirrhosis may be postulated but more information about the metabolism of F VIII protein is needed before such a statement can be proven.

Elevation of F VIII (antihaemophilic factor A, AHF) activity has been reported in a number of physiological and pathological conditions. Extremely high levels have been observed in acute hepatic necrosis due to viral hepatitis [1 6 24 31] but very often an increase of F VIII activity was also found in hepatic cirrhosis [12, 13 32, 37]

Knowledge concerning F VIII has expanded considerably during the past few years. Antisera to highly purified F VIII have permitted to quantify F VIII related antigen (F VIII RA) in plasma. This measurement was of special interest with regard to the nature of the F VIII-deficient states as observed in haemophilia A and von Willebrand's disease [38] Agarose

gel chromatography of F VIII and immunological studies allowed some investigators to propose that F VIII is composed of a high molecular weight carrier protein, which is also identified by immunoprecipitation, and a low molecular weight subunit possessing the coagulant activity [9 10 25 28] This proposal was questioned [23] and recently a unifying model for the synthesis of the F VIII/von Willebrand factor protein was published [11] As the high molecular weight fraction corrected the von Willebrand defect with respect to platelet adhesiveness to glass [7 8] or platelet aggregation in response to ristocetin [35], a quantitative assay of von Willebrand factor was developed [36] Using these newer techniques we measured F VIII activity F VIII RA and von Willebrand factor in 46 patients with hepatic cirrhosis.

Material and Methods

In 46 patients (25 men, 21 women) hepatic cirrhosis was diagnosed on clinical, biological and pathological data. alcoholic cirrhosis in 25, postnecrotic cirrhosis in 15, haemochromatosis with cirrhosis in 2 patients and finally 4 cases of cryptogenic cirrhosis.

38 patients were admitted for decompensation of their cirrhotic process oedema and ascites (28 cases), gastrointestinal haemorrhage (5 cases) and bilirubinaemia higher than 1.5 mg% (30 cases). Some patients were admitted to the hospital several times during the period of our study A blood sample of these patients was examined on each admission, so results of 65 samples are available. Blood samples were also collected from 30 normal volunteers (15 men, 15 women) to determine normal ranges.

Blood samples were collected in siliconized plastic tubes, containing 0.1 vol of 3.8% of sodium citrate. F VIII activity was assayed on fresh plasma with modification of the partial thromboplastin time using kaolin and haemophilia A plasma (F VIII <0.01 U/ml) as substrate. A standard plasma was prepared from pool of citrated plasma from 10 normal subjects. The pooled plasma was said arbitrarily to contain 1.0 U of F VIII activity/ml.

Plasma F VIII RA was measured by the Laurell technique of quantitative immunoelectrophoresis [20]. An antibody to purified F VIII, raised in rabbits, was obtained from Nordic, Tilburg, the Netherlands. Dilutions of the pooled plasma, to construct standard curve, and appropriate dilutions of the test samples were run at the same time. The antigenic material of the pooled plasma was also defined as 1.0 U of F VIII RA/ml.

Von Willebrand factor was assayed with washed human platelets according to Weiss et al. [36]. Washed human platelets were prepared with an albumin density gradient [34]. Ristocetin (H. Lundbeck, Copenhagen) was used in final concentration of 1.25 mg/ml. The pooled normal plasma was considered to contain 1.0 U of

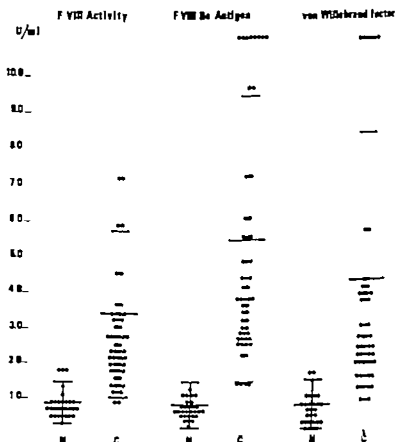


Fig 1 F VIII activity F VIII RA and von Willebrand factor in hepatic cirrhosis (C) and in normal individuals (N). Mean ± SD

von Willebrand factor/ml. A new standard curve was constructed on each day the samples were assayed.

Results

As shown in figure 1 F VIII activity F VIII RA and von Willebrand factor were significantly higher in hepatic cirrhosis than in the controls. Mean values F VIII activity normal 0 88 U/ml, cirrhosis 3.35 U/ml F VIII RA normal 0 80 U/ml cirrhosis 5 42 U/ml von Willebrand factor normal 0 78 U/ml, cirrhosis 4.29 U/ml. These increases were found in decompensated as well as in compensated hepatic cirrhosis no differences

in F VIII activity F VIII RA or von Willebrand factor according to the etiology of the cirrhosis were noted.

A significant correlation between F VIII activity and F VIII RA was found in controls ($r = 0.458$ $p < 0.02$) and in hepatic cirrhosis ($r = 0.5$ $p < 0.001$). Linear relationship between F VIII RA and von Willebrand factor were found in patients with hepatic cirrhosis ($r = 0.338$ $p < 0.01$) and in normal individuals ($r = 0.719$ $p < 0.01$). However in both groups no relationship between F VIII activity and von Willebrand factor was present. F VIII activity was significantly lower than F VIII RA in the group of hepatic cirrhosis ($p < 0.001$). No significant differences were found between F VIII RA and von Willebrand factor nor between F VIII activity and von Willebrand factor

Discussion

Recent studies indicate that antihaemophilic factor A is present in plasma in trace amounts as a large glycoprotein with a molecular weight of approximately 1–2 million daltons [14 19 21 22, 29 30] Immunofluorescence studies, using a monospecific rabbit antihuman AHF showed that endothelial cells contain F VIII RA [4 16] By radioimmunoassay it was demonstrated that cultured human endothelial cells contain F VIII RA which is released into the culture medium [18] Procoagulant activity however was not detected in the culture medium. Although an important progress has been made in the molecular biology of F VIII, the exact role of the liver in the metabolism of AHF is still unknown.

Increased levels of F VIII activity [13 32, 37] and of F VIII RA have been found in liver cirrhosis [12, 17] As in normal individuals F VIII activity and F VIII RA are closely related entities since their levels alter in a parallel manner in a variety of physiological and non-physiological situations [2, 27 39] a significant correlation between F VIII activity and F VIII RA was found in our group of 46 patients. However the levels of F VIII RA were significantly higher than of F VIII activity This was also noted by Green and Ratnoff [12] in 13 of 22 patients with hepatic cirrhosis.

Salt dissociation of AHF procoagulant activity from the antigenic determinants support the concept that F VIII RA and von Willebrand factor are parts of the same protein. Both fluctuate in a parallel manner in normal subjects [36] We found a significant correlation between F VIII

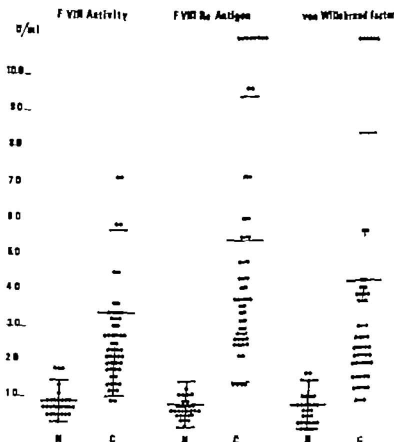


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RA and von Willebrand factor not only in our controls but also in our group of patients with hepatic cirrhosis. However we did not find any relationship between F VIII activity and von Willebrand factor This finding is conflicting with the original report of WEISS *et al* [36] who found a highly significant correlation between von Willebrand factor and F VIII activity This may be due to slight differences in the assay method of von Willebrand factor although we repeatedly found low levels or absence of any correcting activity in the ristocetin induced aggregation of washed normal platelets by plasma of patients with von Willebrand's disease. Finally the mean von Willebrand factor level was higher than the mean F VIII activity in our group of patients with hepatic cirrhosis but this difference was not statistically significant

Procoagulant activity is lost but antigenic determinants are preserved when plasma is treated by thrombin [16, 38] or by plasmin [26] F VIII inactivated procoagulant activity can still support platelet aggregation in response to ristocetin [23] BENNETT *et al* [3] described two patients with premature separation of the placenta and a sharp decrease in the concentration of F VIII activity without corresponding decrease in the level of F VIII RA Inactivation of the procoagulant activity during the course of intravascular coagulation was assumed. A F VIII activity relatively lower than F VIII RA has been observed in some patients with hepatic cirrhosis [12] An identical phenomenon is observed in our group of cirrhotics. However the existence of a low-grade intravascular coagulation in hepatic cirrhosis has been the subject of controversy [5 33] The interpretation of the high levels of F VIII activity RA and von Willebrand factor in hepatic cirrhosis can only be speculative until more information is available about the metabolism of F VIII protein.

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Chronic Myelogenous Leukemia with Elevated Leukocyte Alkaline Phosphatase, Positive Indirect Coombs' Test, Neutrophilic Leukocytosis and Unusual Cytogenetical Findings

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Key Words Chromosomal translocation Chronic myelogenous leukemia Coombs test Leukocyte alkaline phosphatase Neutrophilic leukemia Philadelphia chromosome Thrombocytosis

Abstract An unusual case of chronic myelogenous leukemia (CML) is reported which was characterized by leukocytosis without a shift to the left, elevated leukocyte alkaline phosphatase, positive indirect Coombs test, anemia and thrombocytosis, as well as the absence of hepatosplenomegaly The diagnosis of CML was ascertained by the presence of Philadelphia chromosome with translocation of its deleted arms on the short arms of a chromosome No. 6. The possible relationship between the chromosomal aberration and the unusual hematological and clinical features of this case is discussed.

For almost 20 years it has been generally accepted that in chronic myelogenous leukemia (CML) the leukocyte alkaline phosphatase (LAP) score is substantially decreased [18] While a few cases with elevated LAP scores have been reported all of these were associated with other concomitant diseases [12, 20 28] or with myelofibrosis [8] Cases of CML with neutrophilic leukocytosis [13 22–24] or erythrocyte autoantibodies [16 25] have also been reported but rarely Recently we encountered a case of CML presenting all these features. The diagnosis of CML was based on the presence of Philadelphia (Ph') chromosome with a new translocation on the short arms of chromosome 6 recently described by

us [17]. Following is a detailed description of the clinical and hematological findings in this case.

Case Report

A 42-year-old housewife was hospitalised in February 1974 because of malaise, pruritus, loss of weight and vague bone pains that had started 4 months earlier. Physical examination showed pale-looking, middle-aged woman with a mild parasternal systolic murmur and scratches all over the trunk and extremities. The liver was palpable 1–2 cm below the costal margin, the spleen was not palpable and there was no lymphadenopathy. X-rays of the chest and upper gastrointestinal tract were normal, as were scans of the liver and spleen, which showed no enlargement.

Laboratory examinations of serum glucose, urea, electrolytes, calcium phosphorus, alkaline phosphatase, uric acid, total protein, albumin, globulin, bilirubin, serum glutamic oxalacetic transaminase and lactic dehydrogenase were all within the normal range. Stool guaiac test was negative for occult blood.

Hematologic studies: erythrocyte sedimentation rate 60 mm in the first hour, hemoglobin 9.1 g%, hematocrit 29.9%, erythrocytes 4.05 million/µl, reticulocytes 2.2%, MCV 74 µm³, MCH 22.4 pg, MCHC 30.1 %, platelets 1,400,000/µl and white blood cells 16,000/µl. Differential count: 61% neutrophilic polymorphonuclears, 5% band forms, 21% lymphocytes, 3% monocytes, 2% eosinophils, 8% basophils. The red cells showed anisocytosis, poikilocytosis, hypochromasia, polychromasia and a few spherocytes. One-stage prothrombin determination was 70%. Bleeding and clotting times were normal. Red blood cell survival was within the normal range. Serum iron 117 µg%, iron binding capacity 602 µg%, iron saturation 19%. Serum vitamin B_{12} 1,495 pg/ml; haptoglobin 280 mg%, hemopexin level normal. Coombs' test direct negative, indirect positive (1:8 titer). G6PD was deficient and hemoglobin electrophoresis was normal. Repeated leukocyte alkaline phosphatase determinations performed according to the score method of KAPLOW [14] before initiation of treatment gave consistently elevated values ranging from 190 to 305 (normal 20–70). Bone marrow aspiration showed marked activity mostly myeloid hyperplasia with myeloid erythroid ratio of 5.1. There was normal number of megacaryocytes and deficient hemosiderin content. Bone marrow biopsy revealed markedly increased number of megacaryocytes as well as marked hypercellularity especially of the myeloid population, but fibrosis was not noted. Chromosomal analysis of the bone marrow revealed Philadelphia chromosome with 22q-/9P+ translocation in all the 40 cells counted, with modal number of 46 (17). A simultaneous chromosomal analysis of peripheral blood leukocytes was negative in nonstimulated venous blood cultures and in the presence of phytohemagglutinin showed normal karyotype.

Treatment with oral iron and busulfan (myleran), 6 mg daily was instituted on April 1, 1974. This dosage was gradually reduced to 2 mg before treatment was discontinued on July 15, 1974. At that time leukocytes were 2,300 µl, platelets 100,000 µl and hemoglobin 11.7 g% (table I). LAP determinations obtained during the treatment period showed temporary decrease to scores within normal range but these later rose again, mostly remaining high. In chromosomal analysis repeat-

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Discussion

The difficulties encountered in differentiating between various myeloproliferative diseases are very well illustrated in the present case. The hematological data obtained prior to the chromosomal studies were noncharacteristic and could be classified as being related to the symptomatology of any of the following myeloproliferative disorders: essential thrombocythemia, spent polycythemia or myeloid fibrosis in its early stage. It was the Ph' chromosome which helped classify this case as CML despite the high LAP score.

Only very rarely are LAP scores found to be elevated in cases of CML. In most cases they are either absent or markedly reduced, only seldom being in the low normal range, although in the blastic crisis they may sometimes rise to normal levels. While the literature contains a few reports of Ph chromosome associated with elevated LAP scores, in all these cases there was also some other disease, e.g. cancer of the lung [28] ankylosing spondylitis, iritis, psoriasis [12] and ulcerative colitis [20] or myelofibrosis [8], which could explain this finding. In our case neither concurrent disease nor myelofibrosis could be demonstrated, and the increased LAP remains unexplained.

The occurrence of autoimmune hemolysis or the finding of autoantibodies in CML has also been described but rarely. Videbaek [25] reported two such patients in whom a positive direct Coombs test became negative following the administration of prednisone; the LAP scores were not noted. Maldonado *et al.* [16] reported a case of autoimmune hemolysis with positive direct and indirect Coombs' test with a nonspecific warm panagglutinin and a positive cold hemagglutinin titer in whom the LAP score was zero; prednisone was effective in controlling the hemolysis but the Coombs test remained positive. These authors presumed that the hemolysis had been initiated by busulfan, to which the patient showed unusual sensitivity. Since increased LAP activity due to active hemolysis has not been reported in the literature, and in view of the zero LAP score of this case of Maldonado *et al.* [16], it would appear that the explanation of the increased LAP in our case cannot be found in the presence of autoantibodies as expressed in the positive Coombs' test.

The finding of neutrophilic leukocytosis with no shift to the left in this case was also an unusual feature. Leukemic proliferation of mature granulocytes has been classified as a special entity and has been termed as neutrophilic leukemia. In the four cases in which cytogenetic studies were

Table I Variations of hemoglobin, platelets, white blood count and LAP values during course of illness in patient with chronic myelogenous leukemia

Date	Hemoglobin	Leukocytes/µl	Platelets × 10³/µl	LAP
2.27 74	9 0	16,000	1,400	
3 4 74	9 1	23 000	–	189
3.26.74	8.5	10,800	1 426	305
Busulfan treatment				
4 2.74	8.3	17 400	1 708	–
4.22.74	12.9	10,700	1 400	80
5 13 74	12.6	6,000	470	93
5.20 74	13 1	6,200	292	32
6. 3 74	12.9	5,000	185	222
6.24 74	11 0	4,300	124	348
7.29 74	11 7	1,300	100	259
12.12.74	12.7	3,200	64	130
4.20.75	11.2	3,600	130	196
7 13 75	11 0	4,500	160	86
10.15 75	11 4	2,900	90	62
11 4 75	11.3	3 900	70	128
1.27 76	11 6	3,700	60	55
3.29 76	12.0	3 100	70	110
5.18.76	12.1	3,200	90	98
6.14 76	12.4	4,300	140	116

[1] LAP = Leukocyte alkaline phosphatase normal range 20–70 (score method).

ed 1½ months after discontinuation of the busulfan treatment, Ph chromosome was still found in 28 of 38 metaphases; ten cells showed a normal karyotype.

The patient was hospitalized three times during the period from August to October 1974 because of urinary tract infections as well as for other intercurrent infections. In the follow-up period her hematological status remained stable (table I). Hemoglobin 12.4 g%, erythrocytes 3 76 million/µl, reticulocytes 2.1%, white blood count 4,300/µl. Differential count: 39% neutrophilic polymorphonuclears, 1% band forms, 53% lymphocytes, 7% monocytes. Platelets 140,000/µl Vitamin B_{12} 618 pg/ml. Scans of the liver and spleen were still normal. However the haptoglobin level dropped to 30-120 mg%, the last result having been 55 mg%. The indirect Coombs test remained positive (titer 1.32). No specificity of the incomplete antibodies could be established. Cold agglutinins were within normal limits (1·4). Serum electrophoresis was normal. Repeated red blood survival test showed a T½ of 24 days (normal ± 28±3). When last seen, 24 months after discontinuation of the treatment, the patient was still in sustained remission and in good health.

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reported, there was no Ph chromosome and the LAP was increased [13, 22–24] The combination of Ph chromosome with an increased LAP raises the possibility of a mixed entity exhibiting the hematological characteristics of both CML and neutrophilic leukemia

The reason for the low LAP activity in CML is not clear It has been attributed by some authors to deletion of that part of the Ph chromosome thought to be the locus of LAP activity [1] This assumption is, however contradicted by the fact that LAP may become normal or even elevated during remission or during the blastic phase of CML, even though Ph chromosome is present in as many as 100% of the dividing bone marrow cells [2, 6, 9] Furthermore, the Ph chromosome has been found to be absent in about 10% of the cases of CML despite low LAP values [3 15 26] A possible explanation for this would be a mutation in the LAP locus unconnected with gross chromosomal changes.

ROWLEY [21] was the first to describe the 22q-/9q+ translocation in nine patients with typical CML. WHANG-PENG *et al* [27] in their work-up of five typical CML Ph positive patients have given us a precise analysis of the breaking points of these two chromosomes. This specific translocation has been ascertained in the majority of the cases investigated In a few cases a different autosome, e.g. chromosome numbers 2, 4 8, 11 22, 17 19 was found to participate in the translocation [4 5 7 10 11 19] It may be assumed that there are considerable submicroscopic changes taking place in both of the chromosomes participating in the translocation [5] These changes are likely to introduce variable kinds of genetic imbalance, depending upon the different chromosomes involved.

It may be concluded that CML may appear in various manifestations, a fact which enhances the difficulties of its differential diagnosis with other myeloproliferative disorders. The unusual association of Ph chromosome, neutrophilic leukocytosis, autoantibodies and an increased LAP score unrelated to a concurrent disease, to the best of our knowledge has not yet been described in the literature. The finding of all these features in a case of CML stresses the importance of the chromosomal studies in establishing the final diagnosis.

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Erythrocyte δ-Aminolaevulinic Acid Dehydratase, Urinary Porphyrins and Porphyrin Precursors in Iron Deficiency Anaemia

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Key Words. δ-ALA dehydratase · Erythrocyte metabolism · Haem synthesis · Iron deficiency anaemia · Porphyrin metabolism · Urine porphyrins

Abstract. In 20 iron deficient patients and 21 normal controls the activity of the enzyme δ-ALA dehydratase of erythrocytes was assayed. In addition the urine porphyrins and porphyrin precursor excretions were measured. It was found that in sideropenic patients the erythrocyte δ-ALA dehydratase activity was almost constantly higher than in normals; the difference of the mean values being statistically significant ($p<0.005$). A significant diminution of δ-ALA ($p<0.0025$) urine excretion was observed, whereas the urine excretion of PBG, CP and UP was found within the normal limits. The results are compared to those reported by other authors.

There is good evidence that in iron deficiency anaemia both globin [21] and haem biosynthesis are disturbed [2, 6]. Thus, various authors confirmed that in this condition the free erythrocyte protoporphyrin (EPP) is increased [8, 14, 16, LYBERATOS *et al.*, unpublished data]. Furthermore, DAGG *et al.* [4] found a similar increase even in sideropenic subjects without obvious iron deficiency anaemia. As for the various enzymic steps in haem biosynthesis the data concerning the erythrocyte δ-aminolaevulinic acid dehydratase (δ-ALAD), the enzyme which condenses two molecules of δ-aminolaevulinic acid (δ-ALA) to the monopyrrole porphobilinogen (PBG), are scanty and rather controversial. There is also a similar disagreement as regards the excretion of porphyrins (uroporphyrin, UP; coproporphyrin, CP) and porphyrin precursors (δ-ALA and PBG).

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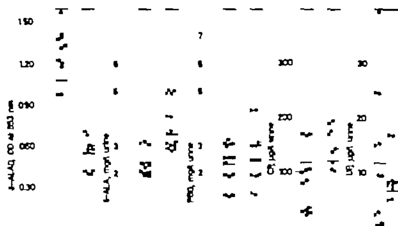


Fig 1 Some parameters of porphyrin metabolism in 20 patients with sideropenic anaemia (●) and 21 controls (O).

cretion is reduced. On the other hand, the urinary PBG UP and CP excretions seem to be unaffected. The pertinent literature is rather limited and considerable disagreement exists. Thus, HEILMEYER [7] in 13 cases and KANEKO [9] in 22 cases found increased erythrocyte δ-ALAD activity On the contrary LICHTMAN and FELDMAN [10] and BATTISTINI *et al.* [1] both in 9 cases had not found significant differences in comparison to normal controls. Furthermore, SHARMA [19] noticed that the enzyme activity in iron deficient rats was increased when expressed as units/ml packed red cells, whereas it remained unchanged when expressed as units/ml of blood. This discrepancy in the results might be attributed to the lower haematocrit in iron deficient rats. A similar discrepancy exists as regards the urinary excretion of δ-ALA. Thus, KANEKO [9] reported a decreased urinary excretion of this substance in 32 sideropenic cases. In contrast, HEILMEYER [7] in 16 sideropenic cases and SHARMA [19] in a number of sideropenic mice found increased urinary δ-ALA excretion. The observed disagreement in the results between various groups of investigators may be due to different methods used.

The mechanism of the increased δ-ALAD activity in sideropenic erythrocytes is at the present difficult to explain. Nevertheless, the enzyme activity could be influenced by the iron concentration. This aspect is supported by the observation of NANDI and SHEMIN [13] who found that

In the present study we assayed both the activity of erythrocyte δ-ALAD and urinary excretion of porphyrins and porphyrin precursors in sideropenic patients.

Material and Methods

The subjects studied were 21 haematologically normal subjects with Hb levels above 14 g/100 ml, normal levels of serum iron and normal saturation of total iron binding capacity (TIBC) 20 patients with iron deficiency anaemia. These cases fulfilled all the clinical and laboratory criteria for diagnosis of iron deficiency anaemia, a history of blood loss, Hb value lower than 10.5 g/100 ml, hypochromia, serum iron lower than 50 μg/100 ml, saturation of TIBC below 15% and empty iron stores in bone marrow

The standard haematological determinations were performed as described by DACIE and LEWIS [3] Serum iron was measured according to PETERS *et al.* [15] For estimation of TIBC the method of RAMSAY [17] was used. δ-ALAD activity was assayed by the method of GIBSON *et al* [5] as described by BATTISTINI *et al.* [1] slightly modified [11] The PBG produced after 1 h incubation is expressed in units of optical density (OD) at 553 nm. For urine δ-ALA and PBG estimation the method of MAUZERALL and GRANICK [12] was followed. Urinary porphyrins were measured according to RIMINGTON [18].

Results

The results are presented in figure 1 In normal subjects the mean erythrocyte δ-ALAD activity expressed in units of OD was 0 550±0.166. In sideropenic patients it was 1 088±0 266 This is significantly different from the normal group ($p<0\ 005$) The mean δ-ALA excretion was lower compared to normal controls (normal controls 3 4±1.5 mg/l sideropenic subjects 2.4±0 8 mg/l) there was a statistically significant difference of the means ($p<0\ 025$) The mean values for urinary PBG were 3 05±1 40 mg/l in normal controls and 2.28±1 13 mg/l in patients, but there was no statistically significant difference between the two groups. The mean urinary UP and CP excretions were 7 20±5.30 and 123±64 μg/l in normal controls and 12±10 and 116±77 μg/l in sideropenic patients. These differences were not statistically significant.

Discussion

The present observations implied that in iron deficiency anaemia the erythrocyte δ-ALAD activity is increased whereas the urinary δ-ALA ex

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δ-ALAD activity is modified by iron in a manner depending on the origin of the enzyme preparation. As for the decreased urinary δ-ALA excretion no explanation can be offered

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acetic acid (3:1) mixture using several changes of this fixative. The metaphases were then spread on a cold wet slide and the preparations were dried by flaming, being careful not to overheat. After 1 week aging at room temperature, banding was performed. Two staining methods were carried out: (1) G-banding: the slides were exposed to 0.01% solution of Bactotrypsin (Difco) 1:250, for 6–10 sec prior to staining in 2% Giemsa solution (Merck Art. 9204) in a phosphate buffer solution at pH 6.88 (Merck Art. 7294). (2) Q-banding: the slides were exposed to a 0.5% aqueous solution of quinacrine dihydrochloride (Atebrin 2900 G.T. Gurr), for approximately 5 min. The preparations were observed with an epi-fluorescence condensor III RS fitted Zeiss Photomicroscope III using violet or blue excitation. The detection of Y bodies in interphase nuclei was performed using the same fluorescence method. The chromosomes set of PHA-stimulated lymphocytes from case 2 were separately analyzed. They were cultured in chromosome medium 1A (Gibco) for 72 h prior to preparation. Preparation and staining were performed using the above-described methods.

Case Reports

Case 1. W. P., a retired warehouse worker, born 1899, without own children, was referred on November 5, 1973, to the Basel University Hospital, Department of Internal Medicine, by his family physician for the further diagnosis of hepatosplenomegaly, leukocytosis and thrombopenia. A myeloproliferative syndrome was entertained as the main diagnosis and interpreted as chronic myeloid leukemia.

Hb 13 g%; WBC 16,000/µl with 44% polymorphonuclear neutrophils, 27% nonsegmented neutrophils, 0.5% metamyelocytes, 0.5% myelocytes, 3.0% lymphocytes, 25% monocytes (partly atypical and young forms); reticulocytes 28 /₀₀, erythroblasts 1/200. Platelets 85,000/µl. LAP score 12 (normal 18–100).

Bone marrow biopsy (University of Basel, Institute of Pathology): hyperplastic marrow with an almost complete displacement of the fat tissue, consisting almost completely of myeloid elements (half segment forms and half immature precursors). Erythropoiesis rarely seen, megakaryocytes not noticeably increased, no fibrosis.

Laparoscopy: both lobes of the liver were distinctly enlarged, the lower edge of the right lobe approximately 8 cm below the right costal margin. The consistency was increased. The spleen was markedly enlarged, the lower pole 1–2 cm below the left costal margin.

Liver biopsy (University of Basel, Institute of Pathology): portal and centroacinar fibrosis. Areas with extramedullary hemopoesis.

Chromosome analysis was performed on November 8, 1973. 20 metaphases from the bone marrow were analyzed. All showed a selective loss of the Y chromosome. One metaphase showed additional loss of several chromosomes which may have been the result of artefacts during the preparation. The chromosomes were well defined and did not show other abnormalities. Also, chromosome 22 looked normal giving no clue for the presence of Ph chromosome.

The patient died 2 years later at home from myocardial infarction as reported by his family physician. No autopsy was performed.

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Loss of the Y Chromosome from Bone Marrow Cells of Males with Myeloproliferative Disorders

Report of Two Cases and Review of the Literature

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Key Words. Chronic myeloid leukemia · Cytogenetics · Karyotype · Myeloproliferative disorders · Philadelphia chromosome · Y chromosome

Abstract. Two additional cases of myeloproliferative disorders are described showing as the only chromosome abnormality a loss of the Y chromosome. Comparing these cases with cases reviewed from the literature indicates that a loss of the Y chromosome in Ph¹-positive and Ph¹-negative CML may cause only a somewhat longer life expectancy following diagnosis. The exact role of the Y chromosome, however, in the initiation or progression of a malignant disorder cannot be stated at this time.

The loss of the Y chromosome has frequently been reported in myeloproliferative disorders and there has been much speculation as to the importance of this loss. In this paper, two patients with myeloproliferative disorders are described in which the affected cells showed a selective loss of the Y chromosome. These and similar cases reported in the literature are compared with respect to the clinical and prognostic meaning of the absence of the Y chromosome in these diseases.

Material and Methods

The bone marrow cells were cultured for 2 and 4 h at 37 °C in McCoy's 5A medium (Gibco) supplemented with 20% fetal calf serum. Mitoses were arrested by adding Colcemid (Ciba) at a final concentration of 1 µg/ml for 2 h. After centrifugation, the cells were suspended in 0.075 *M* KCl for 10 min and fixed in an alcohol/

Table I. Ph¹-negative chronic myeloid leukemias missing Y chromosome

Case No.	Age years	Number of cells studied[1]	Y-negative cells, %	Survival months	Comments	Ref.
Adult form						
1	61	M 85	100	24	blast crisis	55
		B 48	100 (46, XY)		7 months	
2	69	M 65	100	over 18	no blast crisis	17
		B 50	12			
3	74	M 20	100	24	death due to myocardial infarction	case 1
4	79	M 15	100	over 12	osteomyelo-fibrosis/CML?	case 2
		B 20	40			
Juvenile form						
5	3	M 51	96	12½	blast crisis	15
		B 53	56		3 months	

M = bone marrow B = peripheral blood.

characterized by loss of the Y chromosome are listed, whereby two groups are distinguished depending on the presence or absence of the Ph chromosome. Due to insufficient information, the cases mentioned by DE GROUCHY *et al.* [12] (1 case); WHANG-PENG *et al.* [54] (1 case); TOUGH *et al.* [48] (2 cases), BAUTERS *et al.* [2] (1 case) and GROPPELA *et al.* [13] (3 cases) are not listed. All cases with one exception were adults. In 3-year-old boy missing Y chromosome was found in the bone marrow cells in CML which has to be classified as juvenile form [15]. The loss of the Y chromosome as single chromosome aberration in the affected cell in CML is rare (table I). To date only 2 cases [17, 55] have been reported paralleling the 2 cases reported in this paper. Of the two cases from the literature, one had survival time of 24 months before dying from blastic crisis [55]. The other was still alive at the time of publication, total of 18 months following diagnosis [17].

Discussion

Two patients are reported suffering from a myeloproliferative disorder and showing in the affected bone marrow cells as the single chromosome abnormality a loss of the Y chromosome. The diagnosis in the case of pa-

Case 2 L. A., a textile dying expert, born 1896, father of two daughters, was admitted to the Basel University Hospital on April 24 1975 for bone marrow and liver biopsy because his family physician detected abnormal blood findings (immature forms of white blood cells) The only physical signs were localized ecchymoses on the upper extremities.

Hb 13 4 g% WBC 16,000/μl with 42.5% polymorphonuclear neutrophils, 26% nonsegmented neutrophils, 2% metamyelocytes, 7% myelocytes, 0.5% promyelocytes, 9% blasts, 3.5% eosinophils, 3.0% basophils, 5.0% monocytes and 1.5% lymphocytes; reticulocytes 19 ‰, erythroblasts 11/200. Platelets 180,000/μl, partly abnormal and large. LAP score 44

Bone marrow biopsy (University of Basel, Institute of Pathology): hyperplastic marrow and a shift to the left of the myelopoiesis, groupings of megakaryocytes and a severe diffuse fibrosis.

Liver biopsy (University of Basel Institute of Pathology) extramedullary blood producing areas in accordance with the diagnosis of osteomyelofibrosis.

Chromosome analysis was done on April 24 1975 15 metaphases could be analyzed from the bone marrow sample and 20 from the peripheral blood. All bone marrow cells examined presented as the single defect the loss of the Y chromosome using the quinacrine dihydrochloride staining technique and the remaining chromosomes showed no aberrations. Of the 20 mitosis investigated from the blood, 12 had a normal male karyotype and 8 a loss of the Y chromosome. The Y chromosome, if present, showed a brilliantly fluorescent segment equal to the length of its weakly fluorescent part. In addition, interphase nuclei were stained with quinacrine for the detection of the Y chromosome in 500 bone marrow nuclei with loosely structured nuclear pattern. No Y chromatin could be seen, whereas the remaining condensed nuclei occasionally showed a brightly fluorescent Y chromatin body This is further evidence that the affected cell line carried predominately the loss of the Y chromosome.

The patient is still alive and has been readmitted several times because of severe anemia being treated each time with transfusions of erythrocytes.

Cases Reported in the Literature

The loss of the Y chromosome as a specific chromosome abnormality has been found in various hematological disorders. pernicious anemia [21 24 35] polycythemia vera [20 23, 56] sideroblastic anemia [11 19], aplastic anemia [39], refractory dysplastic anemia [53], thrombocytopenia [37], myelodysplasia [37], monocytosis [18], adolescent myelofibrosis [28], acute myeloid leukemia [40] chronic myeloid leukemia (table I, II).

The number of cases reported for each of these entities is quite small and due to the paucity of the information regarding the absence of the Y chromosome in these disorders, it is impossible to arrive at a definite conclusion concerning the significance of this finding. In contrast to this, several males of various age groups suffering from CML have been observed who lost the Y chromosome in a part of or usually all of the affected bone marrow cells. In tables I and II the CML cases

Table II. (continued)

Case No.	Age years	Number of cells studied	Y-negative cells, %	Survival months	Comments	Ref
24	77	B	100	>120	no crisis	27
25	78	M[1]	100	> 27	no crisis	25

M = bone marrow B = peripheral blood.
Number of cells studied not reported.
Not reported.

tient 1 is a chronic myeloic leukemia (CML) whereas in patient 2 the bone marrow biopsy demonstrating severe fibrosis indicated myelofibrosis, a disorder closely related to CML [8] Due to the paucity of reports of similar cases (table I), it is impossible to make a statement regarding the clinical importance of this finding. The number of CML patients having a Ph chromosome in addition to the loss of the Y chromosome is greater as is shown in table II. Because the Ph¹-negative subgroup has generally been estimated to represent only 10–20% of the total CML cases [30] the loss of the Y chromosome could be an event which does not preferentially affect one of the two subgroups of CML.

Much speculation concerning the role of the Y chromosome has been published in the case reports of both forms of CML (see references listed in tables I and II). In contrast to the general view it appears from our collected data that the CML patients with a missing Y chromosome in the affected cells have at the most only a slightly longer survival time than that found in patients with a Y chromosome [7 42] Furthermore, our data do not prove the hypothesis of Garson and Milligan [9] and of Sandberg and Sakurai [41] that the absence of the Y chromosome protects patients from entering a blastic phase. At least 12 of the patients list ed in tables I and II died in a blastic crisis. This conclusion agrees with the view of Lawler *et al.* [25]

In the discussion concerning the significance of the loss of the Y chromosome in the affected cells, it must be mentioned that a missing Y chromosome in bone marrow cells has been observed with increasing age in nonleukemic males by Pierre and Hoagland [36] In their group of males after the age of 79 years as much as approximately 75% of the sub-

Table II Ph[1]-positive chronic myeloid leukemias (adult form) missing Y chromosome

Case No.	Age years	Number of cells studied[1]	Y-negative cells, %	Survival months	Comments	Ref.
1	27	M[2]	XO/XY = 3 1	13	crisis	29
2	27	M[2]	100	93	crisis	22, 25, 47
3	43	M[2] B[2]	100 100	20	crisis 5 months	22, 25
4	43	M 62 B 61	100 39	31	crisis months	43
5	45	M 211 B 34	100 70	49	no crisis	46
6	45	–[3]	–[3]	33	no crisis	14
7	45	B 58	approx. 50	> 48	no crisis	34
8	47	M 50 B 25	100 50	> 24	no crisis	9
9	51	–[3]	100 (?)	42	crisis	14
10	52	–[3]	100	> 82	no crisis	25
11	53	B 85	62	> 6	no crisis	1
12	53	M 50 B 10	100 20	> 24	no crisis	9
13	57	M 10	23	>120	no crisis	5
14	57	M 81	99	[illegible]1	crisis 5 months	[illegible]5
15	58	M 39	43	approx. 24	no crisis	9
16	58	M 31 B 20	87 none	6	crisis 2 months	32
17	59	–[3]	100	91	crisis 5 months	[illegible]2, 25, 47
18	61	M 74 B 138	99 74	–[3]	identification of the Y chromosome not definite	6
19	64	M 18	100	6	crisis	43
20	64	M 61	100	17	crisis	55
21	69	M 188	50	> [illegible]8	no crisis	[illegible]5
22	72	M 27 B 16	100 31	120	no crisis	6
23	74	M[2]	100	22	no crisis	25

orders changes the histocompatibility properties of the affected cells and thus contributes to their uncontrolled proliferation [31].

The mechanism leading to the loss of the Y chromosome is not yet known. The allusion by HAYATA *et al.* [14] that most of the chromosomal changes found in all forms of human leukemia seems to be related primarily to events taking place at centromeric or paracentric regions where the so-called heterochromatin is located deserves special notice. In murine leukemia [3] a considerable number of metaphases were observed showing bridges connecting the heterochromatin segments of two or more chromosomes. This phenomenon may indicate an increased 'stickiness' of the heterochromatin in leukemic cells leading to mitotic nondisjunction. The human Y chromosome usually carries a considerable amount of heterochromatin and, therefore, may be preferentially prone to involvement in such mitotic errors.

Acknowledgements. We wish to express our sincere appreciation to Drs. K. LUDWIG and A. VON ORELLI for allowing us to see the clinical and hematological data of two reported cases. We are also grateful to the Department of Pathology, University of Basel, for supplying us with the results of the various biopsies.

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jects had a missing Y chromosome in at least some of their marrow cells. The occurrence of a physiological loss of the Y chromosome with increasing age is also supported by investigations on bone marrow cells by O'Riordan *et al* [33] by Walker [45] by Sandberg and Hossfeld [42] and on peripheral blood by the Edinburgh group – see review by Court Brown [4] In view of these observations two conclusions can be made: (1) In general the loss of the Y chromosome in bone marrow cells is not an event which is consistently related to malignancy as is the case with the Ph^1 chromosome (2) The degree of hypoploidy varies considerably in the bone marrow cells of nonleukemic males listed as having a loss of the Y chromosome [35] however in CML patients often *all* of the bone marrow cells analyzed were Y-negative (table I II) This fact seems to indicate a selective advantage of the neoplastic cells characterized by the loss of the Y chromosome. This suggestion is strongly supported by the fact that the Y loss in all affected cells also occurs in young men with CML. The average age of the patients with Ph^1-positive CML and a missing Y chromosome (table II) was only 55 years. In this age group a possible physiological loss is rare (see above) The hypothesis of the specific loss of the Y chromosome in leukemic cells is supported by the observation of a group of patients suffering from acute myeloblastic leukemia and carrying a presumably balanced translocation between a chromosome No. 8 and a chromosome No. 21 The Y chromosome was missing in 4 out of 6 such patients [14] In addition, an X chromosome was missing in the only female patient [58]

Loss of the Y chromosome has not only been reported in myeloproliferative disorders but also in other neoplasias such as bronchogenic carcinoma [49] carcinoma of the larynx acute lymphatic leukemia [44] epithelial tumors [49] and several other neoplasias [42]

Cancer is a cellular phenomenon related primarily to a disturbance of the mechanisms regulating cell growth and differentiation. However the fine events causing this disturbance are not yet known [31] Therefore, it is impossible to present a hypothesis for the specific role of the Y chromosome in these events. Actually to date only very little is known about the general function of the Y chromosome except its importance for the development of the male testes. In the mouse, the Y chromosome carries a histocompatibility gene [10] Recently it could be shown that the product of this gene responsible for the H Y antigen has been highly conserved in evolution and is present also in man [50–52] Therefore, it can be theorized that the loss of the Y chromosome in myeloproliferative dis

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B and T Cell Markers of Bone Marrow and Peripheral Blood Lymphoid Cells in Patients with Paraproteinaemia

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Abstract. Studies have been carried out on B and T cells in bone marrow and peripheral blood from patients with paraproteinaemia. The peripheral blood of patients with multiple myeloma showed significant increase of B cells, mainly lymphoid cells bearing immunoglobulins corresponding to the paraprotein, while in patients with benign monoclonal gammopathy only slight increase of B cells and a moderate decrease of T cells have been found. As to the bone marrow, the B cell population was significantly raised in patients with multiple myeloma, but it remained unchanged in patients with benign monoclonal gammopathy. Our findings may offer a new possibility to distinguish between these two diseases and provide further data to their pathogenesis.

Recent developments in immunology afford to obtain more and more information on diseases of the lymphoid system. Investigations of B and T lymphocyte markers from the peripheral blood of patients with paraproteinaemia revealed an increase in the number of lymphocytes bearing heavy or light chains of paraproteins [1, 2, 13, 15, 19, 30]. Most probably, owing to the small quantity of aspirates the study of bone marrow B and T cell markers is less common. Our main purpose was therefore to examine the relative amounts of the lymphoid cells bearing IgG, IgA, IgM, IgD, IgE and C3 as well as those of sheep and mouse erythrocytes forming rosettes with bone marrow (BM) and peripheral blood (PB) lymphocytes in patients with paraproteinaemia.

Materials and Methods

20 patients, 12 women and 8 men aged 40–72, have been studied. In 9 cases (No. 1–9) with multiple myeloma (MM), the diagnosis was established on the basis of agar and immunoelectrophoresis, X-ray analysis of bones, morphology of BM smears by Wright-Giemsa staining, and of other laboratory analyses, e.g., increased erythrocyte sedimentation, anaemia and secondary immunodeficiency. The diagnosis of benign monoclonal gammopathy (BMG) in the remaining 11 patients under observation for 6–36 months (No. 10–20) was based on the criteria usually requested [13, 18, 22] except for those concerning distribution pattern of B and T cells (paraprotein concentration below 2 g/100 ml, no secondary immunodeficiency, no osteolysis, no increase in BM plasma cells). Ten patients (5 women and 5 men aged 30–70), all hospitalized with non-immunological diseases, were selected as controls for BM aspirates after their lymphoid system had been found intact by clinical and laboratory investigations. The PB of healthy staff members (8 women and 7 men aged between 18 and 60) served as blood control.

Lymphoid cell rich suspensions from BM and PB were obtained by density gradient centrifugation as described by Böyum [6]. The PB samples contained 93–98% lymphocytes, while in the BM samples the small and large lymphocyte, lymphoblast and plasma cell contents ranged between 85 and 93%. Trypan blue viabilities of washed cell suspensions were greater than 97%.

Comparative studies, by Parinos [26] on differentiation of B and T mouse lymphocytes have proved that the air-dried smear method is equivalent to the cell suspension technique. For technical reasons the former method was chosen and the immunoglobulin-(Ig) and C3-bearing lymphocytes on unfixed smears were determined by a direct immunofluorescent staining procedure as described in a previous publication [9]. The conjugates contained both heavy and light chain antibodies. At least 400 cells were counted in each case. The relative percentages of both membrane-associated and intracytoplasmic Ig- and C3-positive lymphocytes were determined by examination of the microscope field alternately in blue (480–490 nm) narrow band epi-illumination for fluorescence and in conventional light of a Leitz-Orthoplan microscope (type 860187).

The E rosette-forming ability of lymphocytes (T cells) with sheep erythrocytes was investigated according to the method of Lay *et al.* [21], with some modification [12]. The examinations of mouse erythrocyte rosette-forming (MERF) lymphocytes were performed as published elsewhere [12].

Results

A summary of the normal values obtained is presented in table I. A significant correlation ($p<0.01$) between the total number of Ig- and C3 bearing cells in the BM of controls has been noted. The total percentage occurrence of Ig-coated lymphocytes in the PB of controls showed a high-

Table I Normal values of B and T cell markers from peripheral blood and bone marrow

		Percentages of cells bearing IgG	IgA	IgM	IgD	IgE	C3	total Ig	MERF cells %	E rosettes, %
BM	10									
Mean		6.6	3.5	6.2	3.2	3.1	5.9	22.6	32.2	30.2
± SD		3 0	1.3	2.3	1.8	1.6	3.2	6.1	7 1	9 7
Range		2.7 12.7	1.5–5.2	3.5–9 7	1.2 7.5	1.2–6.7	2 12	15–35	23–44	16–44
PB	15									
Mean		4.9	2.0	5.0	3.6	2.1	5.4	17.6	22.6	68.4
± SD		2.2	1 1	2.3	2.0	1 1	2.0	5.7	4.4	5.9
Range		2 10	0–4 2	2.5–10	0.7–8	0.5–4.2	2.5–8	9 7–28	16–31	57–77

= Number of control individuals examined; E rosettes = T cells forming spontaneous rosettes with sheep red blood cells; MERF cells = lymphocytes capable of forming spontaneous rosettes with mouse erythrocytes; total Ig-bearing cells = sum of percentages of IgG-, IgA-, IgM IgD- and IgE-bearing lymphoid cells.

Table II Ig-bearing cells in patients with paraproteinaemia

Patient No.	Diagnosis	Type of paraprotein	Percentages of Ig-bearing lymphoid cells from BM IgG	BM IgA	BM IgM	BM IgD	BM IgE	PB IgG	PB IgA	PB IgM	PB IgD	PB IgE
1	MM	IgGλ	25.0	5.5	7.7	2.5	8.25	ND	ND	ND	ND	ND
2	MM	IgGκ	43.75	4.5	5.0	7.75	2.0	ND	ND	ND	ND	ND
3	MM	IgGκ	13.25	8.0	7.25	4.5	2.5	19.25	1.5	3.5	1.25	2.0
4	MM	IgGκ	25.0	3.0	4.25	4.0	3.0	16.5	2.0	1.25	1.75	1.5
5	MM	IgGλ	25.0	3.0	4.25	4.0	3.0	36.0	2.25	2.5	0.75	1.0
6	MM	IgGκ	46.0	2.5	6.0	4.75	4.25	21.0	2.25	1.75	1.75	3.0
7	MM	IgGκ	27.0	1.0	1.5	0.5	1.0	34.25	1.25	1.0	2.25	2.5
8	MM	IgGλ	41.0	1.0	1.5	2.0	2.0	27.0	2.0	2.5	2.75	2.5
Mean			30.7	3.6	4.7	3.7	3.2	25.7	2.1	2.1	1.7	1.6
±SD			11.5	2.4	2.3	2.2	2.2	8.1	0.7	0.9	0.7	1.0
$p_1 <$			0.001	NS	NS	NS	NS	0.001	NS	0.01	0.05	NS
9	MM	IgAλ	14.0	18.0	12.0	2.0	2.0	4.0	14.0	8.5	4.5	3.5
10	BMG	IgGκ	15.0	8.0	6.0	7.0	2.0	17.0	3.0	12.5	2.5	1.5
11	BMG	IgGκ	10.0	2.5	6.0	5.0	8.0	18.0	5.5	6.5	7.0	10.0
12	BMG	IgGκ	7.5	5.5	5.0	3.0	6.0	5.0	2.0	5.0	1.5	1.0
13	BMG	IgGλ	6.0	6.5	5.0	3.0	3.25	19.5	3.25	6.25	2.5	2.75
14	BMG	IgGκ	9.5	2.25	4.5	10.5	4.75	15.0	3.75	6.5	24.5	10.5
15	BMG	IgGκ	4.25	1.75	3.0	1.9	0.75	16.75	1.25	3.25	1.0	5.0
16	BMG	IgGλ	5.0	1.0	4.5	3.5	1.25	12.5	2.5	5.75	1.0	2.0
17	BMG	IgGλ	12.0	2.0	4.5	1.0	1.0	6.0	0.5	3.0	15.0	1.5
Mean			8.5	3.7	4.8	4.2	5.0	13.7	2.5	6.1	6.9	4.3
±SD			3.8	2.6	1.0	3.2	4.4	5.9	1.2	3.0	8.8	4.1
$p_1 <$			NS	NS	NS	NS	NS	0.001	NS	NS	NS	0.05
$p_2 <$			0.001	NS	NS	NS	NS	0.02	NS	0.01	NS	NS
18	BMG	IgA+IgM	3.5	7.5	6.0	6.0	4.5	6.5	13.25	16.0	5.0	2.0
19	BMG	IgMκ	4.0	1.0	3.5	1.0	1.5	ND	ND	ND	ND	ND
20	BMG	IgAκ	ND	ND	ND	ND	ND	8.5	12.0	3.0	4.5	4.0

ND = Not determined NS = not significant p_1 = significant difference between the examined markers of patients and controls p_2 = significant difference betwee

Table III. B and T cell markers in patients

Patient No	Diagnosis	Percentages of B and T cell markers from BM: E rosettes	BM: MERF cells	BM: C3-bearing cells	BM: total Ig-bearing cells	PB: E rosettes	PB: MERF cells	PB: C3-bearing cells	PB: total Ig-bearing cells
1	MM	17	24	23.5	48.75	ND	ND	ND	ND
2	MM	27	25	18.5	63.0	ND	ND	ND	ND
3	MM	32	25	18.25	33.5	55	42	12.0	27.5
4	MM	24	ND	11.0	39.25	63	27	18.0	23.0
5	MM	27	ND	11.0	39.25	ND	ND	12.5	42.5
6	MM	29	17	12.0	63.5	62	ND	12.0	29.75
7	MM	50	ND	13.0	31.0	64	12	11.25	41.25
8	MM	13	24	14.0	47.5	63	18	7.0	36.75
9	MM	44	17	27.0	48.0	72	10	20.0	34.5
Mean		29.2	22.0	16.5	46.2	63.2	1.8	13.2	33.3
± SD		11.8	3.9	5.8	11.4	5.4	13.1	4.4	7.4
$p_1 <$		NS	0.02	0.001	0.001	NS	NS	0.001	0.001
10	BMG	18	ND	18.0	38.0	60	ND	11.0	36.5
11	BMG	ND	ND	5.0	31.5	57	ND	6.0	47.0
12	BMG	ND	ND	11.0	27.0	ND	ND	4.0	14.5
13	BMG	42	26	12.55	23.75	56	24	16.5	34.25
14	BMG	33	39	5.0	31.5	58	20	26.5	60.25
15	BMG	46	32	1.25	10.75	66	24	4.75	27.35
16	BMG	30	5	6.25	15.25	64	26	18.0	24.25
17	BMG	18	25	10.25	20.5	70	9	4.25	26.0
18	BMG	ND	ND	4.0	27.5	ND	ND	17.5	42.75
19	BMG	35	ND	3.0	11.0	ND	ND	ND	ND
20	BMG	ND	ND	ND	ND	ND	ND	2.5	34.5
Mean		31.7	25.4	7.6	24.8	61.5	20.6	11.4	34.7
± SD		10.8	12.7	5.1	9.7	5.6	7.0	8.2	12.1
$p_1 <$		NS	NS	NS	NS	0.02	NS	0.05	0.001
$p_2 <$		NS	NS	0.01	0.001	NS	NS	NS	NS

Explanations as in table II.

ly significant correlation with that of MERF cells [12] Distribution pat terns of examined markers in patients with paraproteinaemia are shown in tables II and III.

Biopsy of BM in patients with MM (No 1–9) In the cases of IgG para proteinaemia a significant rise in the frequency of IgG bearing lymphoid cells was observed, while the distribution of other Ig-bearing cells remained normal. The relative amount of IgA-bearing cells increased in the patient with IgA myeloma and the number of IgG- and IgM-bearing cells were higher as well No significant change in the T cell population could be revealed, at the same time the number of MERF cells decreased and the total number of Ig-bearing cells increased significantly

Biopsy of BM in patients with BMG (No 10–20). There was no significant difference between the number of examined cell markers of patients and that of controls

Investigations of PB in patients with MM (No 1–9) Both the increase in the number of IgG-bearing lymphocytes and the drop of amount of IgM and IgD-bearing cells proved significant. Remarkable differences in the number of IgA and IgE bearing lymphocytes, E rosettes and MERF cells were not found. As to the total number of Ig-bearing cells and lymphocytes with membrane-bound C3 a significant enhancement compared to the controls could be detected.

Investigations of PB in patients with BMG (No 10–20). The percentage of lymphocytes bearing Ig corresponding to the paraprotein increased moderately in almost every case, but the mean values of other type of Ig-bearing lymphocytes remained within the normal range. The relative amount of Ig-coated lymphocytes was lower in patients with BMG than that of patients with MM ($p<0.02$). Difference in the number of MERF cells was not revealed between the healthy controls and the patients with BMG The total percentages of Ig- and C3-bearing lymphocytes increased, while the ratio of E rosettes lowered significantly A relatively high number of IgD- and IgE-coated lymphocytes was observed in the 14th case. This patient had hybrid (both atopic and allergic contact) dermatitis, which has been reported previously [10]

Discussion

Our normal values obtained by the examination of B and T cell markers from PB are about the same as or somewhat lower than those gener

ally mentioned in the literature [3 4 9 15 16, 20 22, 27] Relatively fewer data are available about B and T cell markers from BM [1 5 14 17 19 20 23 25] It has been firmly established that mature T lymphocytes do exist within the BM, too. According to our results the percentage occurrence of T lymphocytes in the BM is the same as [17] or higher than that obtained in other studies [5 14] while the total number of Ig-coated cells is remarkably close to the literature data. Preponderance of lymphoid cells (almost 50%) without detectable surface markers has been found in the BM of control individuals.

The percentage of lymphoid cells with cytoplasmic Ig is high, at the same time a low number of lymphocytes carrying membrane Ig can also be detected in the BM of patients with MM [20] Therefore, both types of lymphoid cells have been dealt with in this investigation.

A significantly lowered percentage of B cells having normal surface Ig was found in the myeloma group by LINDSTRÖM *et al.* [22] whereas the patients with BMG showed normal proportions of B cells. Our finding in these two groups exhibit the same results regarding either the BM or the PB. In patients with MM the relative number of IgM and IgD-bearing lymphocytes significantly decreases in the PB, thus revealing a damage in the normal pathway of B cell differentiation [8, 13] In the patients with BMG, a slight increase of B cells and a moderate decrease of T cells have been found in the PB. Whether this phenomenon might be considered as one of earliest signs of the well-known transformation from BMG to MM remains to be seen [13 28] Our study on patients with paraproteinaemia supports the hypothesis that MM is a neoplasia of already differentiated Ig-secreting cells localized in the BM [28] as well as in the PB and that BMG is a disease with low proliferation of B cells which may occur in the PB

Patients with MM demonstrated impaired antibody formation for antigenic stimulation, depressed polyclonal (non-paraprotein) Ig levels in their serums [7 11 13] However in contrast to MM cases the Ig-secreting cells seem to be intact in patients with BMG [18] On the other hand there has been no marked difference in the number of T cells from BM in the patients studied. The total number of Ig-bearing cells (B cells) has significantly increased in patients with MM. Therefore, we can conclude that the rise in the B cell population of BM occurs mainly at the expense of O cells (U cells). The very fact that the number of MERF cells (which are B cell markers [12]) decreases in the BM of patients with MM and does not change in the PB in both diseases suggests that these B cells are malignant

cells different from those obtained in chronic lymphocytic leukaemia [12, 24 29]

The *presumption that the C3-bearing property of certain lymphoid* cells (first published by CORMANE *et al* [9]) might be a new B cell marker seems to be supported by a significant correlation ($p<0\ 01$) between the numbers of C3- and total Ig-bearing cells in the BM of the control group. The EAC rosette-forming cells differ from C3-bearing cells. While the former markers contain C3 receptors with anti-C3 property on the cell membrane the C3-bearing cells can be detected by anti-C3 conjugate, i e C3 can be *found in the cytoplasm and on the membrane produced* and/or carried by the cell. It is hoped that the applications of the technique applied in this work will further facilitate the classification of all patients with paraproteinaemia and lead to a better understanding of the nature of these diseases.

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Lymphocyte Reactivity in Healthy Subjects and Cancer Patients

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Key Words. DNA synthesis · Immunological competence · Immunosuppression in cancer · Lymphocyte stimulation · Malignant tumors

Abstract. Lymphocytes of 40 cancer patients with solid tumors as well as of 40 normal subjects were stimulated by phytohemagglutinin (PHA), purified protein derivative (PPD), streptokinase-streptodornase (SK-SD) and candidin (Cand) and the extent of their stimulation was expressed by the index of reactivity. The cumulative frequency distribution of reactivity indices was significantly higher in normal individuals than in cancer patients. The highest extent of information relevant to the discrimination between patients and normal subjects is obtained following stimulation of lymphocytes with Cand and in decreasing order with PPD, SK-SD and PHA. The percentage of normal subjects and patients having reactive lymphocytes was found to be the highest following stimulation with PHA, lower with PPD and the lowest to Cand and SK-SD. The importance of using increasing numbers of stimulants to achieve maximal detection of lymphocyte reactivity is documented and discussed.

Immune depression has been reported in patients with malignant neoplasia in advanced stages [1-6, 9]. Therefore, the evaluation of immune response can serve as a tool in the clinical evaluation of cancer patients.

In this work the *in vitro* lymphocyte transformation was employed to assess the immunological competence of cancer patients in comparison with normal subjects. The following agents were used as lymphocyte stimulants: purified protein derivative (PPD); streptokinase-streptodornase

With statistical assistance of M. YADIN of the Faculty of Industrial and Management Engineering.

(SK SD) candidin (Cand) and phytohemagglutinin (PHA) The advantage of using multiple antigenic stimulants to obtain maximal detection of immunocompetence was also investigated.

Materials and Methods

Normal subjects comprised 40 normal volunteers selected at random of both sexes, whose mean age was 45 and age range between 25 and 65 years.

The patient group consisted of 40 patients of both sexes, mean age 45 age range 25–65 years. They were suffering from different inoperable malignant solid tumors (gastrointestinal, lung, mammary carcinoma and malignant melanoma). The investigations performed on these patients were carried out prior to administration of radio- and/or chemotherapy

Preparation of lymphocyte cell suspension. Blood was drawn into tubes containing Heparin and Macrodex (Dextran 70, Pharmacia, Uppsala, Sweden) and mixed. The tubes were placed at 45 angle until erythrocyte sedimentation had taken place (30 min) The leukocyte-rich supernatant was then transferred to sterile tubes and centrifuged at 700 rpm for 7 min. The supernatant was discarded and the cells were suspended in 10 ml medium (M 199), supplemented with antibiotics (100 U penicillin and 100 μg streptomycin/ml) After centrifugation the sediment was resuspended in M 199 containing 15% decomplemented human AB serum to a concentration of 1×10^6 mononuclear cells/2 ml of enriched M 199 which were distributed as such into sterile tubes. Into each of these tubes 0.1 ml of optimal concentration of the following antigens were added (1) PPD was prepared from a lyophilized batch of PPD 292 (Tuberculin Section, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory Weybridge, Surrey England) optimal concentration 50 μg/culture tube (2) SK-SD (Varidase, Lederle Laboratories Division, Pearl River N Y.) originally devised for intramuscular use, was employed for stimulation of lymphocytes after previous dialysis at a concentration of 40 U SK/10 U SD culture tube, (3) Cand obtained from the Department of Epidemiology Ness Ziona Institute of Biology Israel, was used for stimulation of lymphocytes at a concentration of 1% saline solution. In addition, the lymphocyte cultures were stimulated with 0.05 ml PHA (Wellcome Research Laboratories, Beckenham, England). This amount was found by previous calibration experiments to stimulate maximally the lymphocyte culture.

Multiple cultures were set up from each of the subjects studied The lymphocytes in the test tubes were stimulated at the same time with each one of the above described agents. The results obtained were related to simultaneously performed unstimulated cultures. The cultures were set up in triplicates and incubated at 37 °C with 5% CO atmospheres for 5 days. On the 4th day of incubation 2 μCi of tritiated thymidine was added to each culture and the cultures were incubated for an additional 18 h. At the end of the labelling period, the cultures were harvested by washing the cells once in ice-cold saline. The nuclear protein was precipitated by 2 ml of ice-cold 5% trichloroacetic acid. The final precipitate was resuspended and passed through a filter disk (Whatman glass paper GF/A) The dried filters were

Table 1 Coefficient of correlation between indices of reactivity of lymphocytes toward different stimulating agents

	Cand	PPD	SK-SD	PHA	
Cand		0.49	0.38	0.24	Patients
PPD	0.17		0.49	0.46	
SK SD	0.15	0.05		0.32	
PHA	0.03	0.16	0.47		
	Normal individuals				

transferred into bottles containing scintillation solution and the radioactivity was measured in Packard liquid scintillation counter. The results obtained were expressed in disintegration per minute (dpm) of the radioactive compound labelling the DNA of the dividing cells. The index of reactivity (stimulation) was calculated from the ratio between dpm of stimulated cultures and dpm of unstimulated cultures. For each test the mean value of triplicate tubes was calculated.

Results

The figures 1–4 represent the cumulative frequency distributions of reactivity indices of lymphocytes toward three memory antigens (PPD SK SD Cand) and PHA for normal subjects and cancer patients. The difference between these two populations is evident especially with antigens toward which a certain population is sensitized following prior contact. The difference was found to be significant at the 0.01 level (by Kolmogorov Smirnov test) for all three memory antigens used and at the 0.01 level for the PHA. Observing the figures 1–4 one may conclude that the highest degree of information relevant to the discrimination between patients and normal subjects is obtained following stimulation of lymphocytes with Cand and a slightly lower degree of information is obtained with PPD The information obtained by stimulating the lymphocytes with SK SD and PHA is of much lower degree than the previous one. As can also be seen in the figures 1–4 the highest percentage of subjects (healthy and sick) having reactive lymphocytes was obtained following stimulation with PHA, a lower percentage with PPD and the lowest with Cand and SK-SD

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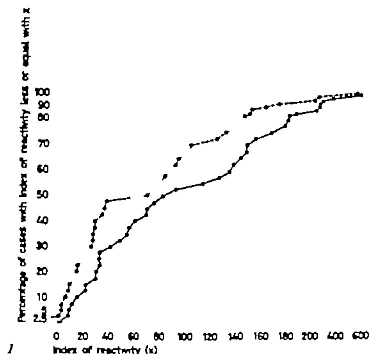
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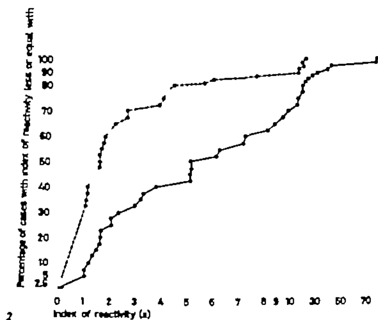
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1



2

Fig. 1, 2. The cumulative frequency distribution of reactivity indices of lymphocytes toward PHA (fig. 1) and toward PPD (fig. 2) —— = Normal controls, - - - = patients.



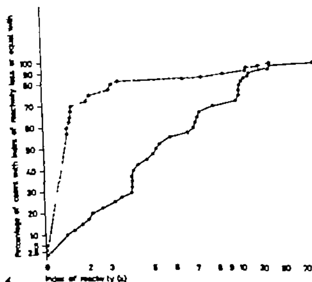


Fig. 3–4. The cumulative frequency distribution of reactivity indices of lymphocytes toward SK SD (fig. 3) and toward Cand (fig. 4). —— = Normal controls, - - = patients.

Table I represents the coefficients of correlation between indices of reactivity of lymphocytes toward the different stimulating agents in the two groups of individuals studied We have found a low positive correlation between indices of reactivity of lymphocytes to different stimulating agents of normal subjects and patients. These coefficients are negligible (and in the case of Cand PHA *even negative) for the normal subjects* with the exception of the coefficient between indices of reactivity of lymphocytes to SK SD and PHA which is 0 47 The coefficients obtained for the patients are somewhat higher (0.3–0.5) and they are significant at 0.02 level. Furthermore, the multiple correlation coefficient between reactivity indices of lymphocytes to PHA and the indices of reactivity of lymphocytes to three memory antigens combined was found to be about 0.5 in both group individuals. These coefficients are significant at the *0 05 level* but once more their value is rather low The fact that correlations between various indices are low indicates low dependency between the results obtained with various stimulating agents. Therefore the information is increasing with the number of stimulating agents used. It does not increase if the results obtained are fully dependent but it is additive whenever the results are fully independent.

Discussion

The lymphocyte transformation to PHA was found to be of prognostic significance since its value decreases in cancer patients with disseminated metastases while it remains in the normal range in localized tumors [6 10 11] It was not yet established whether the cellular immunosuppression detected in cancer patients with solid tumors is the primary cause for the bad prognosis or if it is secondary to the advanced cancer Similarly the lymphocyte transformation to memory antigens was *found to be cor* related with cellular immunity (delayed hypersensitivity skin reaction), and with different stages of malignant diseases [3–5 7 8]

In the present work the lymphocyte transformation to PHA and to three different memory antigens (PPD SK SD and Cand) was applied for evaluation of frequency of lymphocyte reactivity in a group of patients versus a group of normal individuals The differences between these two groups were *found to be significant for all the stimulating* agents used when the highest significance was obtained with Cand and the lowest with PHA. In both groups (healthy and patients) the highest frequency of lym-

phocyte reactivity was obtained to PHA, a lesser frequency to PPD and the least to Cand and SK SD

The high frequency of lymphocyte reactivity to PHA in both groups (100% for healthy individuals and 97.5% for patients) reflects the general capacity of lymphocytes to be nonspecifically stimulated with this potent mitogen. On the other hand the frequency of lymphocyte reactivity to the different memory antigens seems to reflect the sensitization status of a population. This hypothesis was partially substantiated in this work in which it has been proven that by increasing the number of antigens used, an increase in the detection of lymphocyte reactivity was obtained. In addition, technical factors have to be taken into account as well. There are reports in the literature proving that changes in the parameter of lymphocyte transformation test by switching to a semi-micromethod may increase the sensitivity of the test [2]

It was found through statistical analysis that there is low positive correlation between indices of reactivity of lymphocyte to different pairs of stimulating agents. This data suggests that the information obtained following stimulation of lymphocytes with increasing numbers of stimulating agents is additive for the agents studied. Furthermore, there was a positive but low correlation (significant at 0.05 level) between the indices of reactivity of lymphocytes to PHA and indices of reactivity to the three memory antigens combined. Indeed, it was found that only lymphocyte cultures reactive to PHA may be stimulated by memory antigens which may suggest that similar population of lymphocytes may be involved in both cases.

In conclusion this work has proven that all the four stimulating [illegible] are necessary to enable the optimal distribution between lymphocyte [illegible] tivity of cancer patients and healthy individuals. It would be of [illegible] check a larger number of stimulating agents in order to [illegible] the information supplied by them is additive or not. [illegible] of these results and of future experiments being conducted [illegible] tory it will be possible to evaluate more precisely the [illegible] of this test for each particular individual subjected [illegible]

Acknowledgements. W[illegible] are grateful to Mrs. [illegible] nical assistance. This work was supported in part [illegible] of the late LUCIE GUTERMAN administered by the [illegible] or Gesarech Ministry of Justice and the Medical Research [illegible] Ministry of Health.

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Acquired Pyruvate Kinase Deficiency with Hemolysis in Preleukemia

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Key Words. Erythrocyte enzymes · Hemolytic anemia · Preleukemia · Pyruvate kinase deficiency

Abstract. Acquired erythrocyte pyruvate kinase deficiency may appear as symptom secondary to various hematologic disorders, e.g. acute leukemia, sideroblastic anemia, polycythemia vera. The case of a 68-year-old patient with PK deficiency (1.75 U/g Hb) and severe hemolytic anemia is presented, who 1 year later showed acute myeloid leukemia. It is considered that dialysable inhibiting factor may play pathogenetic role in this enzyme change since enzyme activity was raised by dialysis. A survey of the literature is presented.

There have been approximately 150 observations [2, 4, 6, 10–12, 21, 25–27] of erythrocyte pyruvate kinase (PK) deficiency as cause of hereditary nonspherocytic hemolytic anemia [28]. The coincidence of reduced PK activity and acute leukemia was first described by Tanaka *et al.* [25] in two patients: they assumed that in these cases the heterozygote genes of the enzyme defect coincided by chance with leukemia. Since then, acquired forms of PK deficiency have been observed in various leukemias as a secondary symptom [7–9, 16, 18, 20, 23, 26, 27]. Acquired PK deficiency occurs also in other hematologic diseases, e.g. idiopathic and sideroblastic refractory anemia [6–9, 13, 14, 23, 29], panmyelopathy [9, 18], polycythemia vera [9] and myelofibrosis [18].

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Only very few papers support the fact that an acquired PK deficiency can appear as an *early symptom* of leukemia, particularly of acute myeloid leukemia [6 13–16, 23 29] DREYFUS *et al* [14] refer to an anémie réfractaire avec myéloblastose médullaire partielle This particular form of refractory anemia is associated with a low activity in a high percentage of cases (approx. 80–90%) and often turns into acute leukemia [14 16] Acquired PK deficiency may be associated with reduced activity of other enzymes [6–8, 13 14 16 18, 26 27] In these cases, similar as with hereditary heterozygote PK deficiency PK activity is almost invariably reduced to about 60–70% of normal values.

We report the observation of an acquired serious PK deficiency with hemolytic anemia requiring blood transfusions as an early symptom of acute leukemia.

Case Report

A 68-year-old patient who had previously never been seriously ill and who had for years undergone preliminary physical examinations developed hemolytic anemia. Apart from extreme pallor there were initially no pathologic findings, later spleno- and hepatomegaly appeared. The severity of this initially hyperchromic hemolytic anemia varied, necessitating multiple transfusions. The lowest Hb value was 4 4 g%, reticulocytes rose to 90‰. At an early stage there was a leukopenia between 1,700 and 3,200/μl which later disappeared. Thrombocytes were normal. Sedimentation rate 46/94 mm, bilirubin 2.2 mg/100 ml, LDH 228–420 mU/ml, index of alkaline leukocyte phosphatase according to Kaplow 150. Chromosome analysis, carried out to exclude the clinical suspicion of Klinefelter's syndrome, showed nothing extraordinary The cytologically and histologically examined bone marrow was hyperplastic with an excessively increased and morphologically inconspicuous erythropoiesis and an initially maintained granulopoiesis and thrombopoiesis.

One year after the occurrence of anemia the patient was hospitalized with acute myeloid leukemia. In spite of intensive cytostatic therapy according to the VAMP program, only a partial remission of 2 months duration was achieved. At autopsy liver spleen and bone marrow showed acute leukemia. Biochemical investigations indicated a PK deficiency of the erythrocytes. The initial value of enzyme activity was considerably decreased (1.96 or 1 75 U/g Hb. Normal value 8.6±2.8). All other enzyme activities were elevated as a sign of the increased regeneration of the red cell population. The thermostability test of PK [3] indicated an intermediary value of 41 % residual activity after 60 min at 53 °C. During the leukemic stage, the activity of the enzyme was again determined shortly after transfusion. The PK value measured at this time was 4 41 U/g Hb. The activity could be raised by dialysis [1]. The unremarkable family history should be emphasized. Both parents lived for over 80 years without any illness worth mentioning. An alleged healthy sister lives abroad and could not be examined. The patient had no children.

Discussion

An acquired reduction of PK activity as an 'early' symptom of leukemia is not a common finding [6–9 13 14 18, 23 29] It is an epiphenomenon without specific pathogenetic significance for hemoblastoses [1]

Three theories concerning the cause of this form of enzyme change were recently discussed [9] (1) A disturbance of the regulatory mechanism of enzyme activity (2) Either a missing enzyme activator or the presence of a pathologic enzyme inhibitor (3) Damage to genetic material, perhaps chromosomal microlesions. In an acquired PK and phosphofructokinase deficiency ARNOLD *et al.* [1] found a dialyzable, but as yet indefined factor of low molecular weight which acts as enzyme inhibitor In the case we studied, too, the PK activity in the leukemic phase could be raised by means of dialysis by 30%, which is in agreement with the presence of an enzyme inhibitor eliminable by dialysis. In 15 out of 22 cases with low PK activity in acute leukemia or in the early stages of leukemia, a clear increase in enzyme activity could be attained. This confirms, at least in some of the cases, the effectiveness of an enzyme inhibitor Whether the 'acquired enzyme deficiencies' are a uniform group with similar causes remains an open question.

Even in our case it is to be questioned whether the PK deficiency was acquired or hereditary since a PK value as low as 20.3 or 22.8% of the mean normal value, has not yet been described in a 'refractory' preleukemic hemolytic anemia. The lowest estimated PK values in refractory anemia are 32% [29] 37% [7] 37.5% [16] and in acute leukemia 26% [20] It has been pointed out in other studies that PK values below 50% rarely occur in acquired PK deficiency and that transfusions are largely unnecessary [26, 27] The pathogenetic connection between reduced PK activity and hemolytic anemia must remain an open question in the presented case.

The late manifestation of the illness seems less compatible with the hereditary form, although a compliant observation has been reported in a 65-year-old patient [21] The development of hemolytic anemia into acute leukemia likewise points towards an acquired form of PK deficiency However the development of a hereditary PK deficiency into acute monocytic leukemia has recently been reported [17] So far there has been no report of a transformation into acute myeloid leukemia, although as a coincidence this may theoretically be expected. Likewise, the devel-

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Case Report

A 68-year-old patient who had previously never been seriously ill and who had for years undergone preliminary physical examinations developed hemolytic anemia. Apart from extreme pallor there were initially no pathologic findings, later splenoand hepatomegaly appeared. The severity of this initially hyperchromic hemolytic anemia varied, necessitating multiple transfusions. The lowest Hb value was 4.4 g%, reticulocytes rose to 90‰. At an early stage there was a leukopenia between 1 700 and 3,200/μl which later disappeared. Thrombocytes were normal. Sedimentation rate 46/94 mm, bilirubin 2.2 mg/100 ml, LDH 228–420 mU/ml, index of alkaline leukocyte phosphatase according to Kaplow 150 Chromosome analysis, carried out to exclude the clinical suspicion of Klinefelter's syndrome, showed nothing extraordinary The cytologically and histologically examined bone marrow was hyperplastic with an excessively increased and morphologically inconspicuous erythropoiesis and an initially maintained granulopoiesis and thrombopoiesis.

One year after the occurrence of anemia the patient was hospitalized with acute myeloid leukemia. In spite of intensive cytostatic therapy according to the VAMP program, only a partial remission of 2 months duration was achieved. At autopsy liver spleen and bone marrow showed acute leukemia. Biochemical investigations indicated a PK deficiency of the erythrocytes. The initial value of enzyme activity was considerably decreased (1.96 or 1 75 U/g Hb Normal value 8.6±2.8). All other enzyme activities were elevated as a sign of the increased regeneration of the red cell population. The thermostability test of PK [3] indicated an intermediary value of 41% residual activity after 60 min at 53 °C. During the leukemic stage, the activity of the enzyme was again determined shortly after transfusion. The PK value measured at this time was 4 41 U/g Hb The activity could be raised by dialysis [1]. The unremarkable family history should be emphasized. Both parents lived for over 80 years without any illness worth mentioning. An alleged healthy sister lives abroad and could not be examined. The patient had no children.

Discussion

An acquired reduction of PK activity as an 'early' symptom of leukemia is not a common finding [6–9, 13, 14, 18, 23, 29]. It is an epiphenomenon without specific pathogenetic significance for hemoblastoses [1].

Three theories concerning the cause of this form of enzyme change were recently discussed [9]: (1) A disturbance of the regulatory mechanism of enzyme activity. (2) Either a missing enzyme activator or the presence of a pathologic enzyme inhibitor. (3) Damage to genetic material, perhaps chromosomal microlesions. In an acquired PK and phosphofructokinase deficiency ARNOLD *et al.* [1] found a dialysable, but as yet indefined factor of low molecular weight which acts as enzyme inhibitor. In the case we studied, too, the PK activity in the leukemic phase could be raised by means of dialysis by 30%, which is in agreement with the presence of an enzyme inhibitor eliminable by dialysis. In 15 out of 22 cases with low PK activity in acute leukemia or in the early stages of leukemia, a clear increase in enzyme activity could be attained. This confirms, at least in some of the cases, the effectiveness of an enzyme inhibitor. Whether the 'acquired enzyme deficiencies' are a uniform group with similar causes remains an open question.

Even in our case it is to be questioned whether the PK deficiency was acquired or hereditary, since a PK value as low as 20.3 or 22.8% of the mean normal value, has not yet been described in a 'refractory' preleukemic hemolytic anemia. The lowest estimated PK values in refractory anemia are 32% [29], 37% [7], 37.5% [16] and in acute leukemia 26% [20]. It has been pointed out in other studies that PK values below 50% rarely occur in acquired PK deficiency and that transfusions are largely unnecessary [26, 27]. The pathogenetic connection between reduced PK activity and hemolytic anemia must remain an open question in the presented case.

The late manifestation of the illness seems less compatible with the hereditary form, although a compliant observation has been reported in a 65-year-old patient [21]. The development of hemolytic anemia into acute leukemia likewise points towards an acquired form of PK deficiency. However, the development of a hereditary PK deficiency into acute monocytic leukemia has recently been reported [17]. So far, there has been no report of a transformation into acute myeloid leukemia, although as a coincidence this may theoretically be expected. Likewise, the devel-

opment of a hereditary PK deficiency into aplastic anemia has been described as a terminal event in a 71 year-old female patient [4]

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and cryoprecipitates from another larger group of patients were analyzed for antibodies to denaturated DNA (SSDNA) and native DNA (DSDNA) by using a radioimmunoassay method, as well as for DNA by the diphenylamine reaction. All cases have also been examined for the presence of an anti-γ-globulin activity

Materials and Methods

Patients. Samples were obtained from 19 patients with mixed cryoglobulinemia, either essential (9 cases), or secondary to Waldenström's disease (WD) (8 cases) or chronic aggressive hepatitis (CAH) (2 cases).

Sera. Blood was allowed to clot at 37 °C in water bath. Serum was obtained after centrifugation at 800 g for 15 min at 37 °C, it was subsequently inactivated.

Cryocrit. The amount of cryoglobulin in sera was evaluated by the cryocrit, term indicating the volume occupied by the cryoprecipitate, expressed as volume percentage of whole serum after centrifugation for 20 min of serum stored at 4 °C for 72 h, at 800 g and at 4 °C.

Isolation of cryoglobulins. The method described by MELTZER and FRANKLIN [12] was followed.

Characterization of the cryoglobulins. The total protein was measured by the Lowry method [11]. The components of cryoglobulins and of certain supernates were isolated by gel filtration on Sephadex G-200, using 0.1 M acetic acid-acetate buffer pH 3.8.

DNA preparations. Calf thymus DNA (Sigma I highly polymerized) was used as source of DNA preparations. DNA concentrations were determined by measuring the optical density at 260 nm. SSDNA was prepared by heating solutions of 0.5 mg/ml for 12 min in boiling water bath, followed by immediate cooling in an ice bath.

DSDNA was obtained by chromatographic fractionation of solution of 0.1 mg/ml in 0.001 M sodium monophosphate-sodium diphosphate buffer pH 6.8 on a column of hydroxyapatite; elution was accomplished stepwise by increasing gradients in molarity of the same buffer. The fraction eluted by 0.3 M buffer was recovered.

SSDNA was iodinated as described by COMMERFORD [6]. ^{14}C-DSDNA from *Escherichia coli* internally labelled with thymine-^{14}C was obtained from Amersham.

Chemical estimation of DNA was accomplished by the diphenylamine reagent test [14]. Absorbance at 530-595-650 nm was read in Perkin-Helmer spectrophotometer. To eliminate the effects of other sugars the reaction was considered positive only when absorbance data were greatest at 595 nm.

Serologic studies. Anti-γ-globulin activity was measured by the latex agglutination test (Latex Reagent RF Behringwerke) and by the sensitized sheep cell agglutination test (SCAT) [5]. Antibodies to SSDNA and DSDNA were determined by the 50% saturated ammonium sulfate precipitation test originally described by FARR [8] and modified by WOLD *et al.* [18].

Inhibition assay. Specificity of the DNA binding activity was tested in inhibition studies with cold SSDNA and DSDNA by the method of PICAZO and TAN [13].

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Anti γ-Globulin Activity, DNA and Antibodies to DNA in Nonlupoid Cryoglobulinemias

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Key Words. Anti-γ-globulin activity · Chronic aggressive hepatitis · Cryoglobulinemia · DNA antibodies · DNA antigen · Immunoglobulins · Waldenström's disease

Abstract. Anti-γ-globulin activity, free DNA, and DNA binding were studied in 19 cryoglobulinemias: 8 patients with Waldenström's disease (WD), 9 patients with essential cryoglobulinemia (EC), and 2 with chronic aggressive hepatitis (CAH). Antihuman γ-globulin activity was detected in all sera and dissolved cryoprecipitates but two from EC and one from the CAH group. By diphenylamine assay we found DNA in two sera from WD and in one serum and cryoprecipitate from EC. An antibody to denaturated DNA was shown only in sera from the two patients with CAH and from one patient with EC. Nonspecific binding was more frequent.

Cryoprecipitates containing DNA have been demonstrated in patients with systemic lupus erythematosus (SLE) [9] as well as in patients with essential cryoglobulinemia (EC) [4]. Antinuclear antibodies have also been found in cryoprecipitates of SLE patients [3], and DNA antibodies have been reported to be considerably more concentrated relative to serum levels in these cryoprecipitates [7]. In the cases of EC the DNA was firmly bound to serum immunoglobulins although the nature of the interaction could not be determined [4].

In a previous report of our group [2] antibodies to DNA were shown by the hemagglutination technique in a series of patients with nonlupoid cryoglobulinemia; this point is of particular interest because of the possible participation of these antibodies in the formation of cryoprecipitates. In order to assess further the true incidence and the biological significance of these antibodies in mixed nonlupoid cryoglobulinemias, serum

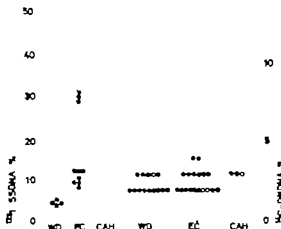


Fig. 2. Percent of ^{125}I SSDNA and ^{14}C-DSDNA bound by sera (O) and cryoprecipitates (●) — Sera which could be inhibited by cold DNA.

Table I. Anti-γ-globulin activity in purified IgG and IgM from cryoprecipitates of patients with WD or EC

Patient	Diagnoses	IgG	IgM
B. M.	WD	0.22	0.0092
C. G	WD		0.0035
P. B.	WD		0.0092
P. M.	WD		0.0064
S. M.	WD	0.065	0.012
C. M.	EC		0.06
C. G	EC		0.027
G. F.	EC		0.04
T. R.	EC		0.01

Minimal agglutinating dose (mg/ml) in the latex agglutination test.

4 cryoprecipitates (1 from WD 3 from EC) and 7 sera (2 from WD 3 from EC, 2 from CAH) revealed SSDNA binding. But this activity was in most instances not due to specific immunoglobulins since it could be inhibited by cold SSDNA only in one EC serum and in both CAH sera, in one of these, native DNA was also found to be an inhibitor (fig. 2). The

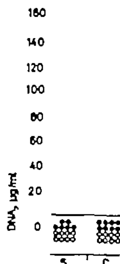


Fig 1 DNA content (µg/ml) in sera (S) and cryoprecipitates (C) of patients with WD (●) or EC (O).

Results

All the cryoglobulins studied were of mixed type. In WD the cryocrit was the most elevated. in two sera its level was over 90%. Of the immunoglobulin components the IgM was always monoclonal of type k, the IgG k and λ in two instances IgA were also present. The amount of cryoprecipitate was smaller in chronic aggressive hepatitis and in essential cryoglobulinemias in both cases it was always composed of monotypic IgM (k) and polyclonal IgG in two cases of EC there were also IgA, in one case only IgG1 and IgG3 Antihuman γ-globulin activity was demonstrated in all sera and dissolved cryoprecipitates from WD patients, in all, but two sera and cryoprecipitates from EC patients, and in one serum from CAH patients. No case presented SCAT positivity The dissolved cryoprecipitate of five sera of WD and four sera of EC were subjected to gel filtration the IgM component had antihuman γ-globulin activity in two cases of WD this activity was also found in IgG (table I). DNA was found in two sera of WD patients. in one its presence was transitory and apparently conditioned by the antiblastic therapy in cryoprecipitates we never found DNA by the diphenylamine reaction. In one patient with EC we detected DNA in serum and cryoprecipitate the ratio µg DNA/mg proteins in serum and cryoprecipitate was similar (12 and 16, respectively) We never found DNA in CAH (fig. 1) When tested for DNA binding,

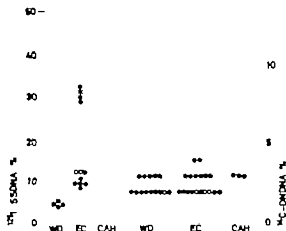


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chromatographic fractionation of these sera showed the SSDNA binding activity in the IgG peak. On the contrary in the purified immunoglobulin fractions obtained from the four cryoprecipitates no binding activity was present. We never found DSDNA binding activity The observed inhibition by DSDNA of antibodies devoid of binding activity to DSDNA may be explained by the different source of DSDNA DSDNA from calf thymus as inhibitor ^{14}C DSDNA from *E coli* in the antigen binding test [15]

Discussion

Our results confirm the close association between WD and antihuman γ-globulin factors, most often identifiable as paraprotein but, at least in some cases, also as the IgG The frequency of this association is less in EC. The finding that in the isolated IgM from WD sera the specific anti-γ-globulin activity studied by examining the minimal latex agglutinating dose of purified protein is higher than in EC suggests that the IgM paraproteins possess an increased avidity and affinity for the antigen.

We have shown the presence of circulating DNA only in a few cases 2 out of 8 in WD 1 out of 9 in EC, never in CAH. Of all the examined cryoprecipitates, we found DNA only in the case of EC. The absence of detectable anti DNA even after fractionation of the cryoprecipitates strongly contrasts the suggestion that our negative results are caused by a blocking of DNA antigen by antibody

We must remember that there is considerable uncertainty as to whether the appearance of circulating DNA is truly pathological A recent report [16] concludes that the presence of DNA in plasma is a pathological event, while in normal serum it is detectable at a concentration of approximately 1.9 μg/ml On this basis we can suggest that the circulating DNA shown in our sera was clearly pathological probably iatrogenic, secondary to alkylating therapy in WD of unknown etiology in EC. Of considerable interest is the finding that the only patient with EC whose serum and cryoprecipitate contained DNA was also suffering from a membranous proliferative glomerulonephritis (bioptically confirmed) On the other hand another patient with a similar glomerular lesion had no demonstrable DNA in her serum and cryoprecipitate.

Specifically inhibitable antibodies to SSDNA were shown in both sera of CAH and in one of EC we never found anti DSDNA activity The absence of circulating DNA in these same sera might be related to an in

termittent antigenemia varying with the clinical course. The failure of demonstrating these antibodies in the cryoprecipitates from the same sera suggests that they are not specifically concentrated during the precipitation process. Therefore this antibody is completely different from that described by WINFIELD *et al.* [17] in SLE cryoprecipitates.

The nonspecificity of SSDNA binding in the great majority of our complexes shown by the absence of inhibition with the cold SSDNA and by the loss of this activity in the isolated fractions suggests a greater than usual positive charge in cryoprecipitating complexes. The SSDNA binding with basic proteins: C1q, collagen, histone has already been described [1, 7 10]. In the patient with EC complicated by glomerulonephritis with DNA in serum and cryoprecipitate without the specific antibody we can suppose a primary localization of DNA in the glomerular basement membrane (10) with subsequent local formation of antigen-antibody complexes. On the basis of our results we can conclude that contrary to SLE the cryoprecipitates in nonlupoid cryoglobulinemias are not composed of DNA and anti-DNA antibodies the possibility exists that other complexes are involved. In the few cases where anti-DNA antibodies are present they are only directed against SSDNA, belong to IgG and are not specifically concentrated in the cryoprecipitate.

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chromatographic fractionation of these sera showed the SSDNA binding activity in the IgG peak. On the contrary in the purified immunoglobulin fractions obtained from the four cryoprecipitates no binding activity was present. We never found DSDNA binding activity The observed inhibition by DSDNA of antibodies devoid of binding activity to DSDNA may be explained by the different source of DSDNA. DSDNA from calf thymus as inhibitor ^{14}C DSDNA from *E coli* in the antigen binding test [15]

Discussion

Our results confirm the close association between WD and antihuman γ-globulin factors, most often identifiable as paraprotein but, at least in some cases, also as the IgG The frequency of this association is less in EC. The finding that in the isolated IgM from WD sera the specific anti-γ-globulin activity studied by examining the minimal latex agglutinating dose of purified protein is higher than in EC suggests that the IgM paraproteins possess an increased avidity and affinity for the antigen

We have shown the presence of circulating DNA only in a few cases. 2 out of 8 in WD 1 out of 9 in EC, never in CAH Of all the examined cryoprecipitates, we found DNA only in the case of EC. The absence of detectable anti DNA even after fractionation of the cryoprecipitates strongly contrasts the suggestion that our negative results are caused by a blocking of DNA antigen by antibody

We must remember that there is considerable uncertainty as to whether the appearance of circulating DNA is truly pathological. A recent report [16] concludes that the presence of DNA in plasma is a pathological event, while in normal serum it is detectable at a concentration of approximately 1.9 μg/ml On this basis we can suggest that the circulating DNA shown in our sera was clearly pathological probably iatrogenic, secondary to alkylating therapy in WD of unknown etiology in EC. Of considerable interest is the finding that the only patient with EC whose serum and cryoprecipitate contained DNA was also suffering from a membranous *proliferative glomerulonephritis (bioptically confirmed)* On the other hand, another patient with a similar glomerular lesion had no demonstrable DNA in her serum and cryoprecipitate.

Specifically inhibitable antibodies to SSDNA were shown in both sera of CAH and in one of EC we never found anti DSDNA activity The absence of circulating DNA in these same sera might be related to an in

termittent antigenemia varying with the clinical course. The failure of demonstrating these antibodies in the cryoprecipitates from the same sera suggests that they are not specifically concentrated during the precipitation process. Therefore this antibody is completely different from that described by WINFIELD *et al.* [17] in SLE cryoprecipitates.

The nonspecificity of SSDNA binding in the great majority of our complexes shown by the absence of inhibition with the cold SSDNA and by the loss of this activity in the isolated fractions suggests a greater than usual positive charge in cryoprecipitating complexes. The SSDNA binding with basic proteins, Clq, collagen, histone has already been described [1, 7 10]. In the patient with EC complicated by glomerulonephritis with DNA in serum and cryoprecipitate without the specific antibody we can suppose a primary localization of DNA in the glomerular basement membrane (10) with subsequent local formation of antigen-antibody complexes. On the basis of our results we can conclude that contrary to SLE the cryoprecipitates in nonlupoid cryoglobulinemias are not composed of DNA and anti-DNA antibodies the possibility exists that other complexes are involved. In the few cases where anti-DNA antibodies are present they are only directed against SSDNA, belong to IgG and are not specifically concentrated in the cryoprecipitate.

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Combined G-6PD and 6-PGD Deficiency in a Hindu Boy

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Key Words. Enzyme erythropathies · Erythrocyte metabolism · G-6PD deficiency · Haemolytic anaemia · Hindu · 6-PGD deficiency · Quinine

Abstract. A case of drug-induced haemolytic anaemia due to G-6PD deficiency is documented because of the associated asymptomatic 6-PGD deficiency – a combination probably reported only once before. Our studies of the patient's family suggest that 6-PGD deficiency is inherited through a simple autosomal dominant gene.

G-6PD deficiency in erythrocytes associated with drug-induced haemolytic anaemia, is now a well-recognised clinico-pathologic entity. Deficiency of another related enzyme in erythrocytes, i.e., 6-PGD, active in the same metabolic pathway (pentose-phosphate) appears to be extremely rare. Less than half a dozen cases have been documented in the literature so far [1]. To the best of our knowledge, deficiency of both G-6PD and 6-PGD in the same individual has been reported only once [2]. We briefly record another such case, most probably the first from this subcontinent.

Case Report

A 5-year-old Hindu boy from Bihar (East India) was referred to us in December 1974 with the following history.

His illness started in August 1973 with a week-long low-grade fever 37.3–37.8 °C. After administration of 30 grains of quinine (total dose 1,375 mg in 3 days) for suspected malaria, this boy passed dark-coloured urine and was hospitalised immediately. A tentative diagnosis of drug-induced haemolytic anaemia due to G-6PD deficiency was made. Quinine was stopped instantly and symptomatic treatment consisting of intravenous glucose-saline infusions and 600 ml of whole blood

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Drug-induced haemolytic anaemias do not seem to occur in individuals heterozygous for 6-PGD deficiency [1], however in two instances where such an association has been documented, there were other metabolic alterations in the anaerobic and aerobic pathways [1]

Acknowledgements. We thank Prof. G J Brewer, Department of Human Genetics, University of Ann Arbor Mich. for his comments on this case and in whose laboratory one of us (S. D.) acquired the familiarity with enzyme assay techniques employed in this study

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Table I Haematological investigations in the patient and his family

	Patient	Father	Mother	First sister	Second sister
Haemoglobin, g%	13 00	14 60	12.80	12.20	12.9
PCV %	42.00	46.00	38 00	–	–
WBC count	7 000	6,500	8,200	–	–
Reticulocytes, %	0 8	0.5	0.5	<1 %	0.6
Plasma haemoglobin, mg%	8 00	7 00	6.00	2.00	4.00
Methaemoglobin reduction	deficient	normal	deficient	deficient	deficient
G-6PD % of normal	6.00	95 00	60.00	75.00	75 00
6-PGD % of normal	50.00	95 00	50.00	98 00	96.00

was given. Complete clearing of urine occurred in 7–8 days. Blood examination prior to treatment revealed. H6 3.6 g%, white cells 8,000/ul with 70% neutrophils, 27% lymphocytes, 2% monocytes, and 1 / eosinophils. Peripheral blood smears and H6 electrophoresis were unremarkable. Urine was positive for haemoglobin.

Haematological investigations of the patient after the haemolytic crisis, and of his parents and two sisters were done in our laboratory The results are summarised in table I. The assays of G-6PD and 6-PGD were done by the methods described by BREWER [1]

Discussion

It will be apparent from the table that the patient had severe G-6PD deficiency and partial 6-PGD deficiency his mother and both sisters were heterozygous for G-6PD deficiency· only the mother was heterozygous for 6-PGD deficiency and the father appeared normal.

According to criteria defined by PARR and FITCH [4] this boy fits the description of the so-called Ilford variant of 6-PGD deficiency in which the enzyme activity is approximately 50% of normal. This is a rare variant and has been explained by the inheritance of a silent PGD gene in association with PGD^A gene with complete penetrance of a simple dominant autosomal gene [3] The most interesting aspect of our case is the co-existence of G-6PD and 6-PGD deficiencies in the same *individual* in which one appears to have been inherited through a sex chromosome and the other through an autosome. Whereas heterozygosity for 6-PGD deficiency results in 50% reduction in enzyme activity that for G-6PD results in variable reduction in the corresponding enzyme activity because of the incomplete penetrance of the offending' gene.

Drug-induced haemolytic anaemias do not seem to occur in individuals heterozygous for 6-PGD deficiency [1] however in two instances where such an association has been documented, there were other metabolic alterations in the anaerobic and aerobic pathways [1]

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Pyridoxal 5-Phosphate-Resistant Sideroblastic Anaemia with Trisomy 8 Mosaicism in the Bone Marrow

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Key Words Cytogenetics Mosaicism Pyridoxal-5-phosphate Sideroblastic anaemia Trisomy 8

Abstract A patient with sideroblastic anaemia manifested a trisomic condition for chromosome number 8 in 75% of his bone marrow cells. Cytogenetic studies of peripheral lymphocytes demonstrated a normal male karyotype. The anaemia was resistant to treatment with pyridoxal 5-phosphate this is in contradiction with the response achieved in some patients with the primary acquired form of sideroblastic anaemia

Ring sideroblasts characteristic of sideroblastic anaemia may result from an undetermined defect in haemoglobin synthesis [1] associated with abnormal pyridoxine metabolism [7] possibly due to an enzymatic defect [12] Pyridoxal 5-phosphate administration [11] has been suggested to alleviate such a possible metabolic defect However various chromosomal abnormalities, including a deletion of chromosome number 20 [2, 4] trisomy 8 [9] heteroploidy [15] and polyploidy [5] have been described in this disorder Such aberrations can induce a variety of cellular defects, including enzyme alterations, which are resistant to pyridoxal [8] We wish to present a second case of sideroblastic anaemia in which bone marrow karyotype revealed mosaicism for trisomy 8 In this case, the treatment with pyridoxal proved unsuccessful

Case History

The patient was admitted on numerous occasions during the 3-year course of his disease He was first examined in August 1973 at age 40 because of a 6-month his

tory of dyspnoea, lassitude, palpitations and ankle oedema. At age 18 he had been informed of a cardiac murmur. Since then, the symptomatology of congestive heart failure developed progressively.

On physical examination, the patient was greyish-pale and showed signs of large atrial septal defect and congestive cardiac failure.

His liver was enlarged 8 cm below the right costal margin and there was no splenomegaly. Haemoglobin 4.5 g/100 ml. The peripheral blood smear showed mostly normochromic erythrocytes with anisocytes and numerous poikilocytes. Some erythrocytes were hypochromic, and there were no nucleated red cells. A bone marrow aspirate was hypercellular with erythroid hyperplasia and with some megaloblasts. Early red cell precursors were disproportionally increased, while many of the mature cells proved to be 'ring' sideroblasts when stained for haemosiderin. The marrow was rich in iron.

Results of biochemical tests were normal except for a serum bilirubin of 1.4 mg/100 ml and a serum glutamic oxaloacetic transaminase of 130 SMA units. Serum iron 228 μg/100 ml, with a saturation of 72%. Serum haptoglobins 250 mg/100 ml, *serum vitamin* B_{12} *760 pg/ml and whole-blood folate 65 ng/ml.* The haematologic and cardiac abnormalities were the only evidence of pathology.

Following transfusions and therapy for congestive heart failure, the patient received the following daily regimen for a period of several months: folic acid (15 mg), pyridoxine (150 mg), oxymetholone (150 mg) and prednisone (30 mg). The drugs were administered singly and in combination. No obvious effect was observed, with the exception of a rise in blood folate to 140 ng/ml, and the diminution of megaloblastic changes in the marrow. Concomitantly the patient required increasingly frequent blood transfusions to maintain a minimum haemoglobin level of 7.0 g/100 ml. His condition was further complicated by increasing evidence of transfusion siderosis, including marked iron deposition in both liver parenchymal and Kupffer cells, as demonstrated by a percutaneous liver biopsy. Daily treatment with 250 mg i.m. of pyridoxal-5-phosphate[1] was instituted in March 1975 for 50 days, with no obvious effect. Therapy was therefore discontinued, with the patient requiring 2 pt of packed red cells approximately every 15 days.

Cytogenetic Studies

Chromosome preparations were obtained from both bone marrow and phytohaemagglutinin (PHA) stimulated and non-stimulated peripheral blood cells. Bone marrow specimens were incubated at 37 °C for 1 h in the presence of Colcemid (demecolcine), 0.05 μg/ml. Peripheral blood was cultured for 72 h with the same concentration Colcemid added for the final hour. All cells were harvested and slides prepared by a slightly modified method of MOORHEAD *et al.* [13], and stained by Giemsa stain. For more precise identification, chromosomal banding patterns were obtained according to the 'Giemsa 9 technique' [14].

A total of 40 metaphases were examined in PHA-stimulated lymphocytes and revealed a modal number of 46 chromosomes with a normal male (46 XY) karyotype.

[1] By courtesy of Roche Products, Welwyn Garden City, Herts.

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His liver was enlarged 8 cm below the right costal margin and there was no splenomegaly. Haemoglobin 4.5 g/100 ml. The peripheral blood smear showed mostly normochromic erythrocytes with anisocytes and numerous poikilocytes. Some erythrocytes were hypochromic, and there were no nucleated red cells. A bone marrow aspirate was hypercellular with erythroid hyperplasia and with some megaloblasts. Early red cell precursors were disproportionally increased, while many of the mature cells proved to be 'ring' sideroblasts when stained for haemosiderin. The marrow was rich in iron.

Results of biochemical tests were normal except for serum bilirubin of 1.4 mg/100 ml and serum glutamic oxaloacetic transaminase of 130 SMA units. Serum iron 228 μg/100 ml, with saturation of 72%. Serum haptoglobins 250 mg/100 ml, serum vitamin B_{12} 760 pg/ml and whole-blood folate 65 g/ml. The haematologic and cardiac abnormalities were the only evidence of pathology.

Following transfusions and therapy for congestive heart failure, the patient received the following daily regimen for period of several months: folic acid (15 mg), pyridoxine (150 mg), oxymetholone (150 mg) and prednisone (30 mg). The drugs were administered singly and in combination. N obvious effect was observed, with the exception of rise in blood folate to 140 ng/ml, and the diminution of megaloblastic changes in the marrow. Concomitantly the patient required increasingly frequent blood transfusions to maintain minimum haemoglobin level of 7.0 g/100 ml. His condition was further complicated by increasing evidence of transfusion siderosis, including marked iron deposition in both liver parenchymal and Kupffer cells, as demonstrated by percutaneous liver biopsy. Daily treatment with 250 mg i.m. of pyridoxal 5-phosphate was instituted in March 1975 for 50 days, with no obvious effect. Therapy was therefore discontinued, with the patient requiring 2 pt of packed red cells approximately every 15 days.

Cytogenetic Studies

Chromosome preparations were obtained from both bone marrow and phytohaemagglutinin (PHA) stimulated and non-stimulated peripheral blood cells. Bone marrow specimens were incubated at 37 C for 1 h in the presence f Colcemid (democolcine), 0.05 μg/ml. Peripheral blood was cultured for 72 h with the same concentration Colcemid added for the final hour. All cells were harvested and slides prepared by slightly modified method of MOORHEAD *et al.* [13], and stained by Giemsa stain. For more precise identification, chromosomal banding patterns were obtained according to the 'Giemsa 9 technique' [14].

A total of 40 metaphases were examined in PHA-stimulated lymphocytes and revealed modal number of 46 chromosomes with normal male (46 XY) karyotype.

By courtesy of Roche Products, Welwyn Garden City Herts.

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Pyridoxal 5-Phosphate-Resistant Sideroblastic Anaemia with Trisomy 8 Mosaicism in the Bone Marrow

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Key Words Cytogenetics Mosaicism Pyridoxal 5-phosphate Sideroblastic anaemia Trisomy 8

Abstract A patient with sideroblastic anaemia manifested a trisomic condition for chromosome number 8 in 75°/ of his bone marrow cells. Cytogenetic studies of peripheral lymphocytes demonstrated a normal male karyotype. The anaemia was resistant to treatment with pyridoxal 5-phosphate· this is in contradiction with the response achieved in some patients with the primary acquired form of sideroblastic anaemia.

Ring sideroblasts characteristic of sideroblastic anaemia may result from an undetermined defect in haemoglobin synthesis [1] associated with abnormal pyridoxine metabolism [7] possibly due to an enzymatic defect [12] Pyridoxal 5-phosphate administration [11] has been suggested to alleviate such a possible metabolic defect However various chromosomal abnormalities, including a deletion of chromosome number 20 [2 4] trisomy 8 [9] heteroploidy [15] and polyploidy [5] have been described in this disorder Such aberrations can induce a variety of cellular defects, including enzyme alterations, which are resistant to pyridoxal [8] We wish to present a second case of sideroblastic anaemia in which bone marrow karyotype revealed mosaicism for trisomy 8 In this case the treatment with pyridoxal proved unsuccessful

Case History

The patient was admitted on numerous occasions during the 3-year course of his disease He was first examined in August 1973 at age 40 because of a 6-month his-

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Case History

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The cytogenetic abnormality in the present case was quite pronounced. The normal karyotype of the peripheral lymphocytes indicates the clonal origin of the cells with chromosomal abnormality [9 10] Treatment with pyridoxine or pyridoxal-5-phosphate had no effect on the degree of anaemia. These features seem to indicate that our patient differed from others with the primary acquired form of the disease, even though he presented no evidence of a myeloproliferative disorder Further experience with similar cases may corroborate the negative predictive value of chromosomal abnormalities for a response to pyridoxal 5-phosphate therapy

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No metaphases were observed in cells cultured without the addition of PHA in the medium. In two bone marrow cultures (January 16, 1975 and March 13, 1975) a mosaic condition was observed. Among the 60 metaphases examined, 45 (75%) revealed 47 chromosomes with an extra member in group C. The remaining 15 cells were normal (46 XY) G-band analysis of the trisomic cells identified the extra chromosome as a member of pair number 8. Examination of a further bone marrow culture, 15 months after the initial one, still revealed the same mosaic trisomy 8 in 15 of 100 metaphases.

Discussion

The acquired sideroblastic anaemias are a heterogenous group of disorders associated with alcoholism autoimmune disorders, haemolytic anaemias, pernicious anaemia lead poisoning treatment with certain drugs or myeloproliferative disorders [12] In some cases, none of the above conditions are implicated and these are referred to as primary acquired sideroblastic anaemias [8 12] Occasionally these patients respond to treatment with pyridoxine or pyridoxal 5 phosphate [6 8 11]

Chromosomal aberrations have been reported in patients with sideroblastic anaemia associated with myeloproliferative disorders and at times, in those with the primary acquired type In the former the cytogenetic abnormalities include trisomy-8 mosaicism [2] heteroploidy [9] polyploidy [15] and F-chromosome deletion [4] Trisomy 9 and 19 and the absence of a G chromosome was observed in patients with the Di Guglielmo syndrome, the early form of which has been equated with acquired sideroblastic anaemia [3] The chromosomal aberrations observed in patients with the primary acquired form consist of a deletion or inversion in the long arm of an F-group chromosome [2, 4]

It has been suggested that the cytogenetic abormalities in patients with primary acquired sideroblastic anaemia result in the enzymatic alteration leading to the defect in haem synthesis [4 6] Some patients respond better to treatment with pyridoxal 5 phosphate than to pyridoxine [6 8 11] It has been postulated that in these cases a decreased activity of pyridoxal kinase effects the conversion of pyridoxine to its active form pyridoxal 5-phosphate [6 11] On the other hand the disturbance in haemoglobin synthesis in sideroblastic anaemia associated with myeloproliferative disorders is believed to be unrelated to pyridoxine and due to disturbed differentiation and maturation of the erythroid cells [3 4] In these cases, the disturbance may result from other enzymatic abnormalies such as decreased haem synthetase or δ-alase activity [3 8]

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A Case of Lambda Type Tetramer Bence-Jones Proteinemia

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Key Words. Bence-Jones proteinemia · Bence-Jones proteinuria · Light chain of immunoglobulin · Plasmacytoma · Tetramer dimer and monomer

Abstract. A 50-year-old woman was found to have an extramedullary plasmacytoma of the thoracic vertebrae and bone marrow plasmacytosis of 72%. In the serum of this myeloma patient, M-bow precipitin line was seen only with anti-lambda chain serum, but not with antisera against mu, alpha, gamma, delta, epsilon and kappa chains. Gel filtration of the serum on the Sephadex G-200 column demonstrated the monoclonal lambda chain in the A-fraction along with transferrin and suggests molecular weight of 88,000 and tetrameric light chain. Bence-Jones protein has never been detected in the patient' urine.

Bence Jones protein had been regarded as a pathognomonic protein found in the urine but not in the serum of patients with myeloma. In 1962, Bence-Jones protein was found to be identical to the light chains of the immunoglobulins [1, 2]. Recent progress in clinical immunochemical analysis has demonstrated Bence-Jones protein not only in the urine but also in the blood plasma.

Bence-Jones proteinemia usually is associated with concomitant Bence-Jones proteinuria [5]. Three cases of tetrameric Bence-Jones proteinemia without Bence Jones proteinuria, however, had been reported since 1964, all of which were lambda type [6–8]. In the present report, a new case of tetramer lambda type Bence Jones proteinemia not associated with Bence-Jones proteinuria is presented.

Materials and Methods

Immunoelectrophoresis was performed according to the method of Scheidegger [9]. Antisera against Bence-Jones proteins (kappa and lambda), Fc, alpha-chain, mu-

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chain and delta-chain were prepared in our laboratory in rabbits by multiple intracutaneous injections of the respective human protein fractions in Freund's adjuvant. Antiserum against IgE was obtained from Hoechst Company. Gel filtration of the Bence Jones proteins and the patient's serum was carried out by use of a Sephadex G 200 column (2.5 × 100) with 0.1 Tris-buffer (pH 8.0) containing 0.15 M sodium chloride. The light chain was isolated from the A fraction by DEAE-cellulose chromatography using 0.01 M phosphate buffer (pH 7.5).

Case Report

A 53-year-old woman, F. F., noted a pain in the left breast in July 1974. Since September 1974 she had a gait disturbance and hypalgesia in the lower extremities. She was admitted to the neurological institute, Kyushu University, and a diagnosis of an epidural tumor of the upper thoracic vertebrae was made.

In October 1974 a laminectomy of the thoracic vertebrae (T4-T7) and partial removal of the tumor were performed. Histologic diagnosis of the extramedullary tumor was plasmacytoma. The patient was referred to our clinic in November 1974. She had no lymphadenopathy and no hepatosplenomegaly, but a hard tumor of the left breast was found. Lower limbs showed muscle weakness and hyperreflexia. Hypesthesia, hypalgesia and thermohypesthesia below the fifth thoracic vertebra were noted.

Hemoglobin was 11.3 g%, RBC 458 × 10⁴/mm³. Blood sedimentation rate was 13 mm in the first hour. Bleeding time was 3 min, prothrombin time 11.8 sec and partial thromboplastin time 59.3 sec. Blood urea nitrogen 6.0 mg%, calcium 9.3 mg%, alkaline phosphatase 136 mU/ml (IU). Isozyme pattern of the serum alkaline phosphatase revealed only fraction III, known to be of bone origin. Urine analysis showed no proteinuria throughout the clinical course.

Kidney function tests, such as concentration test, creatinine clearance and phenolsulfophenyl retention test were normal. The bone marrow pattern showed a remarkable increase of atypical plasma cells: plasma cells 72.4%, reticulum cells 0.4%, lymphocytes 10.8%, monocytes 10.8%. Total bone marrow nucleated cell count was 11.5 × 10⁴/mm³. Bone scintigram by ⁹⁹ᵐTc revealed an abnormally high area of activity in the thoracic and lumbar vertebrae. ⁶⁰Co irradiation was administered in addition to steroids and cyclophosphamide, and the patient gradually improved and was found to be in good condition in December 1974.

Results

Immunochemical studies of the patient's serum protein. Total protein concentration in the serum was 6.9 g% with albumin 62.3%, alpha 1 globulin 3.1%, alpha 2 globulin 10.9%, beta globulin 7.8% and gamma-globulin 15.7%. The gamma-globulin did not increase in concentra

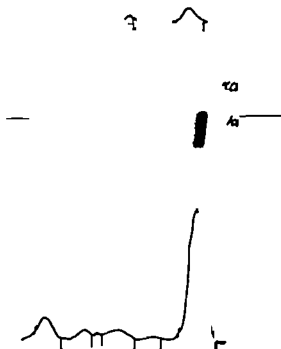


Fig. 1 Electrophoretic patterns on cellulose acetate membrane of the serum (a) and urine (b) of the patient, F. F. The urine was concentrated 200 times than original urine. Urine protein: Alb. 62.8%, α_1 37.1 %. Serum protein: 6.8 g/dl; Alb. 60.9%, 2.8%, α_2 11.8%; β 7.1 %; γ 17.1 %.

tion, but the cellulose acetate electrophoretic pattern of the serum protein revealed a monoclonal peak at the fast gamma-globulin region as shown in figure 1. Immunoelectrophoresis of the serum showed a M-bow precipitin line at the fast gamma-globulin region, which was ellicited only with anti-lambda chain antiserum. No abnormal precipitin lines were produced by using antisera against Fc of IgG, alpha-chain, mu-chain, delta-chain,

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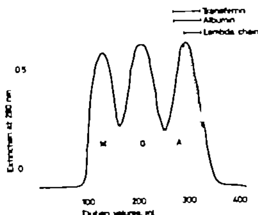


Fig. 3. Gel filtration of the patient' serum (F F) on Sephadex G-200. Eluting solution. 0.1 M Tris-HCl (pH 8.0) containing 0.15 M NaCl.

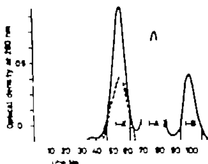


Fig. 4. Gel filtration of Bence-Jones proteins (F F) on Sephadex G-100 (2.5 × 100). Eluting solution: 1 M acetic acid. Fraction Size 3.7 ml. I A = Dimer of the BJ protein, I A monomer of the BJ protein, I-B = the fragment protein of BJ protein ---- = BJ protei from BJ proteinuria (kappa chain, Lo) — = BJ protein from BJ proteinemia (lambda chain, F F)

on the Sephadex G-200 column was carried out, and as shown in figure 3 the lambda type Bence-Jones protein was eluted in the A-fraction along with transferrin which has a molecular weight of 88,000. These findings demonstrated that the lambda type Bence-Jones protein in the myeloma patient's serum was tetramer of Bence-Jones protein in the neutral pH

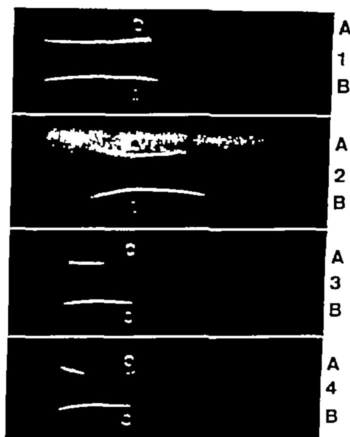


Fig 2 Immunoelectrophoresis of the serum of the patient, F F A = The patient's serum (F F) B = normal human serum 1 = anti Fc (IgG) (contaminated with antitransferrin) = anti-alpha chain 3 = anti kappa chain 4 = anti-lambda chain.

ipsilon-chain and kappa-chain Mancini tests of the serum protein showed IgG 760 mg%, IgA 54 mg%, IgM 38 mg%, IgD less than 20 mg% and IgE less than 20 mg%. 200 times concentrated urine of the patient did not show any Bence-Jones protein in the immunoelectrophoresis. On the basis of these immunochemical studies of the serum protein lambda type Bence Jones protein in the serum without Bence-Jones proteinuria was ascertained (fig 1 2)

Molecular size of the lambda Bence-Jones protein in the patient's serum In order to determine the molecular size of the lambda type Bence Jones protein in the serum of the myeloma patient, F F the gelfiltration

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range, because the monomer of Bence Jones protein has a molecular weight of 22,000 The lambda type Bence-Jones protein (F F), isolated from the A fraction of DEAE-cellulose chromatography however became a dimer as shown in the chromatogram on gel filtration using 1 M acetic acid solution (fig 3 4)

Discussion

Until 1964 it was generally believed that there was no Bence-Jones proteinemia without Bence-Jones proteinuria [5] Rare cases of Bence-Jones proteinemia without Bence-Jones proteinuria, however have been reported since 1964 Four occurrences of verified Bence-Jones tetramers in the serum have been reported, gamma-globulin fragments of the Bence Jones type [6] tetramer Bence Jones proteinemia [7] IgG myeloma protein and tetramer of Bence-Jones protein in the serum [8] and tetramer of Bence Jones protein in the serum and urine [10]

In our patient there was Bence-Jones proteinemia without Bence-Jones proteinuria. It is interesting that all of the reported tetrameric Bence-Jones proteins in the serum are of the lambda type Bence-Jones proteins can exist in three different forms as monomers of 22,000 molecular weight as unstable dimers of 44 000 molecular weight which are noncovalently bonded dimers and are converted into monomers when placed in dissociating agents such as 1 M acetic acid and as stable dimers which are disulfide bondes [11–13] The present case however had tetrameric Bence-Jones protein in the serum, which was converted to dimer in 1 M acetic acid This suggests therefore that the tetramer of Bence-Jones protein in our patient consisted of two stable dimers [7 8 10] In general lambda type Bence-Jones proteins appear to exist as stable dimers and tend to form tetramers, whereas kappa type Bence-Jones proteins are usually present as monomers or unstable dimers [11–13]

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range, because the monomer of Bence-Jones protein has a molecular weight of 22,000 The lambda type Bence-Jones protein (F F) isolated from the A fraction of DEAE-cellulose chromatography however be came a dimer as shown in the chromatogram on gel filtration using 1 M acetic acid solution (fig 3 4)

Discussion

Until 1964 it was generally believed that there was no Bence-Jones proteinemia without Bence-Jones proteinuria [5] Rare cases of Bence Jones proteinemia without Bence-Jones proteinuria however have been *reported since 1964 Four occurrences of verified Bence-Jones tetramers* in the serum have been reported, gamma-globulin fragments of the Ben ce Jones type [6] tetramer Bence-Jones proteinemia [7] IgG myeloma protein and tetramer of Bence-Jones protein in the serum [8] and tetra mer of Bence Jones protein in the serum and urine [10]

In our patient there was Bence Jones proteinemia without Bence-Jones proteinuria It is interesting that all of the reported tetrameric Bence-Jones proteins in the serum are of the lambda type. Bence-Jones proteins can exist in three different forms as monomers of 22,000 molecular weight as unstable dimers of 44 000 molecular weight which are noncon valently bonded dimers and are converted into monomers when placed in dissociating agents such as 1 M acetic acid and as stable dimers which are disulfide bondes [11–13] The present case however had tetrameric *Bence-Jones protein in the serum, which was converted* to dimer in 1 M acetic acid This suggests, therefore, that the tetramer of Bence-Jones protein in our patient consisted of two stable dimers [7 8 10] In general, lambda type Bence-Jones proteins appear to exist as stable dimers and tend to form tetramers, whereas kappa type Bence-Jones proteins are usually present as monomers or unstable dimers [11–13]

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2 PUTNAM F W Structural relationships among human γ-globulin, myeloma globulins and Bence-Jones protein. Biochim biophys. Acta *63* 539 (1962).

Our close friendship began at this time In 1947 we organised together the first meeting of the 'Société Internationale Européenne d'Hématologie in Montreux and afterwards we continued working together for many years in this group.

The main interest of Heini' – if I may be so personal – was applied to functional and clinical Haematology In later years as clinical supervising physician, he still continued to nurture the broad base of medicine. For him haematology was not an end in itself but an integral part of internal medicine and one that should be helpful in diagnosing and determining the patient s illness. Here I wish to point out his exceptional didactic talent which, combined with his typical Basel humour made him one of the best-liked professors among the students and nurses. He was a meticulous and critical researcher Besides his extensive medical knowledge his patients sensed and appreciated his genuine empathy and concern.

As a physician he was especially interested in the education of nurses and was an active teacher at the nursing school of the Bürgerspital. This led to appointments to various bodies of Nursing Commissions. With the same enthusiasm and dedication he availed himself to military service and was awarded the commission of full colonel and corps physician. He also contributed much to the post-graduate education of our Red Cross officers in the army

His interest in haematology was, in the beginning, mainly concerned with the examination of living blood cells with the phase microscope. He paid special attention to sideroblasts and siderocytes. Along with these studies he always developed the excellent microscopic photographs himself the enlargements as well as the slides. Later he turned to serological problems of the haemolytic anaemias.

His habilitational paper 'Organpunktate in der klinischen Medizin written in 1954 is still worth reading. The same applies to the chapter on the Plasmozytom in the Handbuch der Hämatologie

In 1951 he took upon himself the additional responsibility of Secretary of *Acta Haematologica*. Owing to his questioning mind and his extensive knowledge he contributed much to the success of this journal and was appointed Editor-in-Chief of Acta Haematologica In 1962 he was awarded a teaching position as Professor Extraordinary of Haematology at the University of Basel.

It is common knowledge that especially active and highly gifted individuals avail themsel es for the achievement of common goals especially when they possess an excellent talent for organisation. He always found it



In Memory of Professor Heinrich Lüdin

With the death of Prof HEINRICH LÜDIN the field of medicine has lost an eminent hematologist and physician and the University of Basel a distinguished teacher and Chief of the Department of Haematology of the Medical Clinic.

He was born in Basel in 1913 and after completing his medical studies he devoted himself to the area of pathology under the tutelage of Prof WERTHEMANN and subsequently to that of internal medicine with RUDOLF STÄHELIN and HANS STAUB In the latter field he was promoted to the position of Supervising Physician

The undersigned first became acquainted with HEINRICH LÜDIN 30 years ago at a conference of the Swiss Society of Haematology At this time he already impressed us all as a confident lecturer and authority in his field. With the combination of a clear and concise presentation and his talent for conveying his knowledge he left a lasting impression

Index rerum ad Vol. 57

Bearbeitet von G. [illegible], Basel

(B) = Book reviews   Buchbesprechungen   Livres nouveaux

Acid phosphatase   Lysosomes

Agglutinins, cold   agglutinins, in   case of chronic lymphatic leukaemia (A study of the lymphocyte surface) 206

Aggregates of platelets   circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and lauric acids in guinea-pigs 87

AHG A,   Factor VIII

Alkaline phosphatase, elevated (leukocytes)   Myelogenous leukaemia, chronic

Alpha-thalassaemia   interaction of haemoglobin E with α-thalassaemia and haemoglobin Constant Spring 109

Aminolaevulinic acid dehydratase (δ),   Delta-aminolaevulinic dehydratase

Anaemia, haemolytic   congenital Heinz body haemolytic anaemia due to haemoglobin Perth in an Asian child seemingly aggravated by the high nitrate content of the water supply 143

glucose-6-phosphate dehydrogenase Velletri (A new variant with reduced activity in   patient with congenital non-spherocytic haemolytic anaemia) 121

Haemoglobin E, Haemolysis, Phosphogluconate dehydrogenase, Pyruvate kinase deficiency

Anaemia, haemolytic, non-spherocytic, chronic,   Erythrocyte membrane

Anaemia, hyperproliferative   sideroblast score   A sensitive indicator of iron deficiency and hypoproliferative anaemia 24

Anaemia, iron deficiency   erythrocyte δ-aminolaevulinic acid dehydratase, urinary porphyrins and porphyrin precursors in iron deficiency anaemia 305

Dipyridamole

Anaemia, mediterranean,   Thalassaemia

Anaemia, megaloblastic (vitamin B_{12} or folate deficiency),   Megaloblastic erythropoiesis

Anaemia, sideroblastic   idiopathic acquired refractory sideroblastic anaemia Banded chromosome analysis in six patients 15

pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354

Anaemia, hypoproliferative   sideroblast score   A sensitive indicator of iron deficiency and hypoproliferative anaemia 24

Anaemia, sideroblastic   idiopathic acquired refractory sideroblastic anaemia   banded chromosome analysis in six patients 15

Anaemia

Angio-immunoblastic lymphadenopathy   Myelofibrosis

Anovulant drugs,   Pyruvate kinase deficiency

most difficult to say no if someone asked him for favour still in the face of all this work and responsibilities he remained a very humble person In spite of the busy working schedule he always found time for his patients colleagues and friends and most of all for his wife and family A love for the beauty of nature and classical music were a welcome and soothing relaxation to him.

Suddenly nine years ago his untiring efforts and career were interrupted by a massive heart attack. It seems almost miraculous that, thanks to modern and intensive care and the efforts of all his colleagues, he recuperated to such an extent as to allow him to return to his duties at the clinic as supervisor of the Haematological Department.

We all share the sorrow in the loss of a good friend an outstanding physician and human being as well as an exceptional clinical haematologist. His professional expertise will leave a lasting impression upon the many colleagues and numerous nurses, to whom he was able to transmit a deeper understanding for patients, and above all his outstanding knowledge of internal medicine, specially in the area of clinical haematology

SVEN MOESCHLIN *Solothurn*

The Publishing House and Dr h c. THOMAS KARGER wish to express their appreciation to Prof MOESCHLIN for his moving obituary and add that they lose, with the passing of Prof LÜDIN not only an excellent and highly esteemend editor but also a personal friend. The relationship with Prof LÜDIN spans two generations of the Karger family and we shall keep him in fond memory

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Index rerum ad Vol. 57

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Acid phosphatase, Lysosomes

Agglutinins, cold agglutinins, in a case of chronic lymphatic leukaemia (A study of the lymphocyte surface) 206

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Aminolaevulinic acid dehydratase (δ), Delta-aminolaevulinic dehydratase

Anaemia, haemolytic congenital Heinz body haemolytic anaemia due to haemoglobin Perth in an Asian child seemingly aggravated by the high nitrate content of the water supply 143

glucose-6-phosphate dehydrogenase Velletri (A new variant with reduced activity in patient with congenital non-spherocytic haemolytic anaemia) 121

Haemoglobin E, Haemolysis, Phosphogluconate dehydrogenase, Pyruvat kinase deficiency

Anaemia, haemolytic, non-spherocytic, chronic, Erythrocyte membrane

Anaemia, hyperproliferative sideroblast score A sensitive indicator of iron deficiency and hypoproliferative anaemia 24

Anaemia, iron deficiency erythrocyte δ-aminolaevulinic acid dehydratase, urinary porphyrins and porphyrin precursors in iron deficiency anaemia 305

Dipyridamole

Anaemia, mediterranean, Thalassaemia

Anaemia, megaloblastic (vitamin B_{12} or folate deficiency), Megaloblastic erythropoiesis

Anaemia, sideroblastic idiopathic acquired refractory sideroblastic anaemia Banded chromosome analysis in six patients 15

pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354

Anemia, hypoproliferative sideroblast score A sensitive indicator of iron deficiency and hypoproliferative anemia 24

Anemia, sideroblastic idiopathic acquired refractory sideroblastic anemia banded chromosome analysis in six patients 15

Anaem

Angio-immunoblastic lymphadenopathy Myelofibrosis

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sistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354
-v. Anaemia, sideroblastic· B and T cell markers Erythropoiesis, megaloblastic·
Bone marrow cells of males loss of the Y chromosome from bone marrow cells of males with myeloproliferative disorders (Report of two cases and review of the literature) 310
Bone marrow human 128 (B)
Bone marrow iron, v Sideroblast score
Book reviews 127 128 (B), 366 (B)
Buchbesprechungen 127 128 (B), 366 (B)
BURKITT's lymphoma cell leukaemia 188

[14]C, Cryoglobulinaemia
Calcium in platelets a simple and rapid platelet calcium determination (Normal values and results in MAY-HEGGLIN anomaly) 168
Cancer patients lymphocyte reactivity in healthy subjects and cancer patients 331
Cations of erythrocytes, Dipyridamole
Cell cultures, Tetraploid cell line (leukaemia)
Cell surface, Scanning electron microscopic study
Chain (J-chain), Tetrameric light chain
CHEDIAK-HIGASHI syndrome large granules and lysosomal fusion in human CHEDIAK-HIGASHI white blood cells 279
Chemotherapeutic remissions in Wistar/Furth rat acute myelogenous leukaemia A model for human acute myelogenous leukaemia 233
Chemotherapy Hydroxyurea (tumors), Imidazole carboxamide (HODGKIN' disease), Peptichemio (Lymphomas)
Children effect of blood transfusion on the immune response of children with thalassaemia 65
Chromatography Folic-acid-binding protein, Microchromatography
Chromosomal translocation, Myelogenous leukaemia, chronic
Chromosome banding· idiopathic acquired refractory sideroblastic anaemia. Banded chromosome analysis in six patients 15
Chromosomes, v Sideroblastic anaemia, Tetraploid cell line (leukaemia)
Circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135
Cirrhosis, hepatic, v Liver cirrhosis
Coagulation of blood A laboratory manual of blood coagulation 127 (B)
Coagulation of blood, Antithrombin III deficiency Factor VIII, Factor XII, Haemophilia (B), Platelet(s), Platelet aggregates, Postoperative platelet function
Cold agglutinins in case of chronic lymphatic leukaemia (A study of the lymphocyte surface) 206
Complement receptor lymphocytes simplified one-step procedure for the simultaneous determination of complement receptor lymphocytes and lymphocytes with membrane-bound lymphocytes 74
Congenital disorders of erythropoiesis (Ciba Foundation symposium 37 (new series) (1976) 366 (B)
Congenital Heinz body haemolytic anaemia due to haemoglobin Perth in Nepal child seemingly aggravated by the high nitrate content of the water supply 143
Congenital non-spherocytic haemolytic anaemia glucose-6-phosphate dehydrogenase Velletri (A new variant with reduced activity in a patient with congenital non-spherocytic haemolytic anaemia) 121
Congress, Haemophilia (B)
Contraceptives, oral, Pyruvate kinase deficiency
COOMBS' test, indirect, chronic myelogenous leukaemia with elevated leukocyte alkaline phosphatase, positive indirect COOMBS' test, neutrophilic leukocytosis and unusual cytogenetical findings 298
[51]Cr Transferrin-immune

Antibodies, platelet antibodies circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135

Antibodies to DNA anti-γ-globulin activity DNA and antibodies to DNA in nonlupoid cryoglobulinaemia 344

Antibody transferrin antibody v Transferrin-immune

Anticoagulant, circulating, against factor XII and platelet antibodies in systemic lupus erythematosus 135

Antigen, DNA antigen, v DNA (antibodies, antigen)

Antigen, factor VIII-related antigen v Factor VIII

Antigen, leukaemia antigens preparation, purification and *in vitro* properties of a serum against human lymphoblastic leukaemia-associated antigens 257

Anti-γ-globulin activity DNA and antibodies to DNA in nonlupoid cryoglobulinaemia 344

Antihaemophilic factor A activity factor VIII-related antigen and VON WILLEBRAND factor in hepatic cirrhosis 290

Antihaemophilic globulin A, (= AHG A) v Factor VIII

Antileukaemic serum preparation, purification and *in vitro* properties of a serum against human lymphoblastic leukaemia associated antigens 257

Antithrombin III deficiency familial thrombosis due to anti-thrombin III deficiency in a *Greek* family 47

Arachidonic acid circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and lauric acids in guinea pigs 87

Atomic absorption spectrophotometry v Calcium in platelets

Atransferrinaemia transferrin-immune complex disease 96

Autoimmune disease, v Transferrin-immune

Autoradiography high resolution autoradiography electron microscope and high resolution autoradiographic studies of megaloblastic erythropoiesis 1

Banded chromosome analysis idiopathic acquired refractory sideroblastic anaemia Banded chromosome analysis in six patients 15

BARR, v EPSTEIN-

B cells B and T cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321

BENCE JONES proteinaemia a case of lambda type tetramer BENCE JONES proteinaemia 359

Beta-thalassaemia heterozygotes, screening quantitation of Hb A_2, with DE-23 microchromatography in whole blood as screening test for β-thalassaemia heterozygotes 32

Blast cells, tetraploid, v Tetraploid cell line (leukaemia)

Blast transformation, v Lymphocyte reactivity

Blood cells, human, white (CHEDIAK HIGASHI syndrome), v Lysosomes

Blood coagulation, a laboratory manual 127 (B)

Blood coagulation, v Antithrombin III deficiency Factor VIII, Factor XII Haemophilia (B), Platelet(s), Platelet aggregates, Postoperative platelet function

Blood peripheral (lymphoid cells), v B and T cell markers

Blood transfusion effect of blood transfusion on the immune response of children with thalassaemia 65

Blutplättchen, v Platelet(s)

B lymphocytes B and T cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321

B lymphocytes, human, leukaemic, scanning electron microscopic study 247

B lymphocytes, v Antileukaemic serum, Cold agglutinins, Lupus erythematosus

Bone marrow pyridoxal-5-phosphate-re-

erythropoiesis (Ciba Foundation symposium 37 (new series) (1976) 366 (B)
Erythropoiesis, megaloblastic, electron microscope and high resolution autoradiographic studies 1
Extramedullary haemopoiesis histogenesis of myeloid metaplasia in the spleen 81

Factor VIII (= Antihaemophilic globulin A = AHG A) deficiency inherited platelet abnormalities associated with low factor VIII activity in the same family 225
Factor VIII antihaemophilic factor A activity factor VIII-related antigen and von WILLEBRAND factor in hepatic cirrhosis 290
Factor XII (= Hageman factor) circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135
Familial thrombosis due to antithrombin III deficiency in *Greek* family 47
Families, two related inherited erythrocyte pyruvate kinase deficiency Studies on 15 members of two related families 37
Family inherited platelet abnormalities associated with low factor VIII activity in the same family 225
Family *Indian*, haemoglobin Shepherds Bush (β74 [E 18] Gly→Asp) in an *Indian* family 102
Family Platelet abnormalities
Fatty acid-induced platelet aggregation, Lauric acid
*Fe, Transferrin-turnover
Feinstruktur Ultrastructure
Fer Sideroblastic anaemia, Sideroblast score, Transferrin-turnover
Ferment erythropathies, Enzyme erythropathies
Ferments, Delta-aminolaevulinic acid dehydratase, Glucose-6-phosphate dehydrogenase, Neuraminidase, Phosphatase Phosphogluconate dehydrogenase deficiency Pyruvate kinase deficiency
Fine structure, Ultrastructure
Finger-prints, Haemoglobin Perth, Haemoglobin Shepherds Bush
Fluorescing agent (quinacrine mustard), Chromosome banding
Foie, Liver cirrhosis
Folate deficiency Megaloblastic erythropoiesis
Folic-acid-binding protein in normal subjects, study 156
Freeze-fracture of circulating human eosinophils 129

Gammaglobulin anti-γ-globulin activity DNA and antibodies to DNA in non-lupoid cryoglobulinaemia 344
Gammopathy Paraproteinaemia
Gel-filtration, BENCE JONES proteinaemia
Genetics, Congenital Enzyme erythropathies, Families, Family Haemoglobinopathies, Heredopathies, Platelet abnormalities
Ghost enzymes, Erythrocyte membrane
Globulins, Gammaglobulin
Glucose-6-phosphate dehydrogenase (= G6PD) Hamburg membrane deformability of erythrocytes with glucose-6-phosphate Hamburg 162
Glucose-6-phosphate dehydrogenase Valletta (A new variant with reduced activity in patient with congenital non-spherocytic haemolytic anaemia) 121
Glucose-6-phosphate dehydrogenase (= G6PD) and 6-phosphogluconate dehydrogenase (= 6-PGD), combined deficiency in *Hindu* boy 351
Glutathione reductase, Anaemia, sideroblastic
G6PD (= Glucose-6-phosphate dehydrogenase) and 6-PGD (= 6-phosphogluconate dehydrogenase), combined deficiency in a *Hindu* boy 351
Granule membranes, Human eosinophils
Granules, large, and lysosomal fusion in human CHEDIAK-HIGASHI white blood cells 279
Greek family familial thrombosis due to

Cryoglobulinaemia nonlupoid *anti-γ-globulin activity* DNA and antibodies to DNA in nonlupoid cryoglobulinaemia 344
Culture des cellules, v Tetraploid cell line (leukaemia)
Cytochemistry v Lysosomes
Cytogenetic findings, unusual, v Myelogenous leukaemia, chronic
Cytogenetics, v Sideroblastic anaemia, Y chromosome
Cytokinetics, v *Tetraploid cell line (leukaemia)*
Cytostatic therapy v Myelofibrosis
Cytostatics, influence on some platelet functions *in vitro* (V Hydroxyurea) 242
Cytotoxic drugs, v Cytostatics
Cytotoxicity v Antileukaemic serum

Deformability membrane deformability of erythrocytes with glucose-6-phosphate dehydrogenase Hamburg 162
Delta-*aminolaevulinic* acid dehydratase erythrocyte δ-aminolaevulinic acid dehydratase, urinary porphyrins and porphyrin precursors in iron deficiency anaemia 305
Desoxyribonucleic acid, v DNA
Dipyridamole effects on sodium and potassium content of human red blood cells 193
DNA (= Desoxyribonucleic acid) antibodies, *antigen anti-γ-globulin activity* DNA and antibodies to DNA in nonlupoid cryoglobulinaemia 344
DNA synthesis, v Leukaemia, acute Lymphocyte reactivity Megaloblastic erythropoiesis
DTIC, v Imidazole carboxamide

Eisen v Sideroblast score, Sideroblastic anaemia, Transferrin-immune
Electron microscope and high resolution autoradiographic studies of megaloblastic erythropoiesis 1
Electron microscope scanning electron microscopic study of leukaemic human B lymphocytes 247
– v Human eosinophils, Lysosomes
Electrophoresis, v Bence Jones proteinaemia, Haemoglobin E
Enfants, v Children
Enzyme erythropathies, v Erythrocyte-δ-aminolaevulinic acid dehydratase, Erythrocyte membrane (G6PD), Erythrocyte pyruvate kinase deficiency Glucose-6-phosphate dehydrogenase (G6PD), Phosphogluconate dehydrogenase deficiency (6-PDG) Pyruvate kinase deficiency
Enzymes, v Ferments
Eosinophils freeze-fracture of circulating human eosinophils 129
Epstein-Barr virus, v Infectious mononucleosis
Erythematodes, v Lupus erythematosus
Erythrocyte δ-aminolaevulinic acid dehydratase, urinary porphyrins and porphyrin precursors in iron deficiency anaemia 305
Erythrocyte membrane membrane deformability of erythrocytes with glucose-6-phosphate dehydrogenase Hamburg 162
– *v Erythrocytes of rabbits*
Erythrocyte pyruvate kinase deficiency Adverse effect of oral contraceptives 116
Erythrocyte pyruvate kinase deficiency acquired, with haemolysis in preleukaemia 339
Erythrocyte pyruvate kinase deficiency inherited Studies on 15 members of two related families 37
Erythrocytes, metabolism effects of dipyridamole on sodium *and potassium* content of human red blood cells 193
– v Enzyme erythropathies
Erythrocytes of rabbits, life span *in vivo* behaviour of neuraminidase-treated rab*bit erythrocytes and reticulocytes* 178
Erythropathies, enzyme erythropathies, v Enzyme erythropathies
Erythropoiesis congenital disorders of

pathies, Haemoglobinopathies, Haemophilia (B), Platelet abnormalities, Thalassaemia
Heterozygotes, β-thalassaemia heterozygotes, Haemoglobin A_2, quantitation
HIGASHI, CHEDIAK ...
High resolution autoradiography Autoradiography
Hindu boy: combined G6PD and 6-PGD deficiency in a *Hindu* boy 351
Histochemistry Cytochemistry
³H-leucine, v. Megaloblastic erythropoiesis
HODGKIN's disease: Imidazole carboxamide (DTIC) in the treatment of advanced lymphomas (Efficacy of DTIC in cases which fail to respond to conventional chemotherapeutic combinations) 272
Infectious mononucleosis in HODGKIN's disease (A further case report) 152
HODGKIN, Non-HODGKIN, lymphomas
³H-thymidine, Megaloblastic erythropoiesis
Human acute myelogenous leukaemia, model, v. Rats, Wistar/Furth rats (acute myelogenous leukaemia)
Human B lymphocytes, leukaemic, scanning electron microscopic study 247
Human bone marrow 128 (B)
Human eosinophils, circulating, freeze-fracture 129
Human leukaemic cells, antileukaemic serum preparation, purification and *in vitro* properties of serum against human lymphoblastic leukaemia-associated antigens 257
Human red blood cells: effects of dipyridamole on sodium and potassium content of human red blood cells 193
Human white blood cells (CHEDIAK-HIGASHI syndrome): large granules and lysosome fusion in human CHEDIAK-HIGASHI white blood cells 279
³H-uridine, Megaloblastic erythropoiesis
Hypoproliferative anaemia: sideroblast score. A sensitive indicator of iron deficiency and hypoproliferative anaemia 24
Hydrea® v. Hydroxyurea
Hydroxyurea (=Hydrea®): influence of cytostatics on some platelet functions *in vitro* (V. Hydroxyurea) 42
Hypersiderinaemia, v. Transferrin-immune
¹²⁵I v. Cryoglobulinaemia
Idiopathic acquired refractory sideroblastic anaemia. Banded chromosome analysis in six patients 15
Idiopathic myelofibrosis, chronic, Myelofibrosis
IgM, Cold agglutinins
Ig (M-Ig), Immunoglobulins, membrane-bound
Imidazole carboxamide (DTIC) in the treatment of advanced lymphomas (Efficacy of DTIC in cases which fail to respond to conventional chemotherapeutic combinations) 272
Immune complex disease: transferrin-immune complex disease 96
Immune response: effect of blood transfusion on the immune response of children with thalassaemia 65
Immunoelectrophoresis, BENCE JONES proteinaemia
Immunoglobulin, BENCE JONES proteinaemia
Immunoglobulin-bearing cells, Lupus erythematosus
Immunoglobulins, membrane-bound; simplified one-step procedure for the simultaneous determination of complement receptor lymphocytes and lymphocytes with membrane-bound immunoglobulins 74
Immunoglobulins, BENCE JONES proteinaemia, Cold agglutinins, Gammaglobulin, Paraproteinaemia
Immunological competence, Lymphocyte reactivity
Immunosuppression in cancer Lymphocyte reactivity
Infectious mononucleosis in HODGKIN's disease (A further case report) 152

antithrombin III deficiency in a *Greek* family 47
Guinea-pigs circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and lauric acids in guinea-pigs 87

Haarzell-Leukämie, v Scanning electron microscopic study
Haem synthesis, v Porphyrins, urinary
Haematology quality control in haematology (Symposium of the International Committee for Standardization in Haematology) 128 (B)
Haemochromatosis, v Anaemia, sideroblastic Transferrin-immuno
Haemoglobin A_2, quantitation with DE – 52 microchromatography in whole blood as screening test for β-thalassaemia heterozygotes 32
Haemoglobin Constant Spring interaction of haemoglobin E with α-thalassaemia and haemoglobin Constant Spring 109
Haemoglobin E, interaction with α-thalassaemia and haemoglobin Constant Spring 109
Haemoglobin Perth, nitrate effect congenital Heinz body haemolytic anaemia due to haemoglobin Perth in a *Nama* child seemingly aggravated by the high nitrate content of the water supply 143
Haemoglobin Shepherds Bush (β74 [E 18] Gly→Asp) in a *Italian* family 102
Haemoglobinopathies, v Haemoglobin A_2, Haemoglobin Constant Spring, Haemoglobin Perth, Haemoglobin Shepherds Bush, Thalassaemia
Haemolysis acquired pyruvate kinase deficiency with haemolysis in preleukaemia 339
– producing agents of acute haemolysis, v Myeloid metaplasia
Haemolytic anaemia congenital Heinz body haemolytic anaemia due to haemoglobin Perth in a *Nama* child seemingly aggravated by the high nitrate content of the water supply 143
– glucose-6-phosphate dehydrogenase Velletri (A new variant with reduced activity in a patient with congenital non-spherocytic haemolytic anaemia) 121
– v Haemoglobin E, Phosphogluconate dehydrogenase Pyruvate kinase deficiency
Haemolytic anaemia, non-spherocytic, chronic, v Erythrocyte, membrane
Haemophilia (Proceedings of the IXth Congress of the World Federation of Haemophilia (1974)), 366 (B)
Haemophilia, v *Factor VIII*
Haemopoiesis, extramedullary histogenesis of myeloid metaplasia in the spleen 81
Hageman factor v Factor XII
Hairy cell leukaemia, v Scanning electron microscopic study
Hb A_2 v Haemoglobin A_2 (quantitation)
Hb E, v Haemoglobin E
Hb Shepherds Bush, v Haemoglobin Shepherds Bush
HEGGLIN, v MAY
HEINZ bodies, v Deformability (erythrocytes)
HEINZ body haemolytic anaemia, congenital, due to haemoglobin Perth in a *Nama* child seemingly aggravated by the high nitrate content of the water supply 143
Hemochromatosis, v Anemia, sideroblastic
– v Haemo
Hemolysis acquired pyruvate kinase deficiency with hemolysis in preleukaemia 339
– v Haemol
Hepar v Liver cirrhosis
Heparin postoperative platelet function in patients on small subcutaneous doses of heparin 266
– v Folic-acid-binding protein
Heparin cofactor v Antithrombin III
Hepatic cirrhosis, v Liver cirrhosis
Hepatitis, aggressive, chronic, v Cryoglobulinaemia
Heredopathies, v Antithrombin *III* deficiency Congenital Enzyme erythro-

A model for human acute myelogenous leukaemia 233
Leukemia, myelogenous, chronic with elevated leukocyte alkaline phosphatase, positive indirect Coombs's test, neutrophilic leukocytosis and unusual cytogenetical findings 298
Leukemia, Leukaemia(s)
Leukemic B lymphocytes, human, scanning electron microscopic study 247
Leukemic, Leukaemic.
Leukocyte alkaline phosphatase, elevated, Myelogenous leukaemia, chronic
Leukocyte lysates, Folic-acid-binding protein
Libri 127 128 (B), 366 (B)
Lien, Spleen
Light chain of immunoglobulin case of lambda type tetramer Bence Jones proteinaemia 359
Liver cirrhosis antihaemophilic factor A activity factor VIII-related antigen and von Willebrand factor in hepatic cirrhosis 290
Livres nouveaux 127 128 (B), 366 (B)
Lüdin, Heinrich, Prof. † Editor-in-Chief of *Acta haematologica*, in memoriam, 366
Lupus, drug-induced, Lupus erythematosus
Lupus erythematosus, systemic circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135
Lupus erythematosus, systemic, peripheral blood lymphocytes (Relation to activity) 199
Lupus erythematosus, Nonlipoid cryoglobulinaemia
Lymphadenopathy angio-immunoblastic, Myelofibrosis
Lymphatic leukaemia, chronic, cold agglutinins in case (A study of the lymphocyte surface) 206
Lymphoblastic leukaemia, human preparation, purification and *in vitro* properties of a serum against human lymphoblastic antigens 257
Lymphocyte reactivity in healthy subjects and cancer patients 331
Lymphocyte stimulation, Lymphocyte reactivity
Lymphocyte surface markers cold agglutinins in a case of chronic lymphatic leukaemia (A study of the lymphocyte surface) 206
Lymphocytes simplified one-step procedure for the simultaneous determination of complement receptor lymphocytes and lymphocytes with membrane-bound immunoglobulins 74
B lymphocytes, leukaemic, human, scanning electron microscopic study 247
–,B and T cell markers of bone marrow and peripheral lymphoid cells in patients with paraproteinaemia 321
Lymphocytes of peripheral blood in systemic lupus erythematosus (Relation to activity) 199
Lymphogranulomatosis maligna, Hodgkin's disease
Lymphoma, malignant Burkitt's lymphoma cell leukaemia 188
Lymphomas, malignant imidazole carboxamide (DTIC) in the treatment of advanced lymphomas (Efficacy of DTIC in cases which fail to respond to conventional chemotherapeutic combinations) 272
'v Scanning electron microscopic study...
Lymphomas, malignant (non Hodgkin lymphomas) Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211
Lymphosarcoma, Imidazole carboxamide
Lysosomes large granules and lysosomal fusion in human Chediak-Higashi white blood cells 279

Inherited platelet abnormalities associated with low factor VIII activity in the same family 225
In memory of Prof HEINRICH LÜDIN Editor-in-Chief of *Acta haematologica* 366
International Committee for Standardization in Haematology Symposium Quality control in haematology 128 (B)
Iron, v Sideroblast score, Sideroblastic anaemia, Transferrin-immune
Iron deficiency anaemia, v Anaemia, iron deficiency Dipyridamole
Iron overload, v Transferrin-immune
Iron staining of bone marrow smears, v Sideroblast score
Isotope radioaktive, v Cryoglobulinaemia, Megaloblastic erythropoiesis, Transferrin-immune
Italian family haemoglobin Shepherds Bush (β74 [E 18] Gly→Asp) in an *Italian* family 102

K, v Potassium
Kälte Agglutinine v Cold agglutinins
Karyotype, v Sideroblastic anaemia, Tetraploid cell line (leukaemia)
Karyotype, v Sideroblastic anaemia
Kationen der Erythrozyten v Dipyridamole
Kinder v Children
Knochenmark, v Bone marrow
Komplement, v Complement receptor lymphocytes
Kongenital, v Congenital
Kryoglobulinaemie, v Cryoglobulinaemia

Laboratory manual of blood coagulation 127 (B)
Lambda chain, v Tetrameric light chain
Lauric acid circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and lauric acids in guinea-pigs 87
Leber v Liver cirrhosis
Leucine (^{3}H), v Megaloblastic erythropoiesis
Leukaemia, acute, atypical tetraploid cell line in a girl with acute leukaemia 55
Leukaemia antigens preparation, purification and *in vitro* properties of a against human lymphoblastic leukaemia associated antigens 257
Leukaemia BURKITT's lymphoma cell leukaemia 188
Leukaemia, lymphatic, chronic, cold agglutinins in a case (A study of the lymphocyte surface) 206
Leukaemia lymphoblastic, human preparation, purification and *in vitro* properties of a serum against human lymphoblastic leukaemia-associated antigens 257
Leukaemia, myelogenous, acute, in rat chemotherapeutic remissions in Wistar/Furth rat acute myelogenous leukaemia A model for human acute myelogenous leukaemia 233
Leukaemia, myelogenous, chronic, with elevated leukocyte alkaline phosphatase, positive indirect COOMBS's test, neutrophilic leukocytosis and unusual cytogenetical findings 298
Leukaemia, myeloid, chronic loss of the Y chromosome from bone marrow cells of males with myeloproliferative disorders (Report of two cases with review of the literature) 310
Leukaemic B lymphocytes, human, scanning electron microscopic study 247
Leukemia BURKITT's lymphoma cell leukemia 188
Leukemia antigens preparation, purification and *in vitro* properties of a serum against human lymphoblastic leukemia-associated antigens 257
Leukemia, lymphoblastic, human preparation, purification and *in vitro* properties of a serum against human lymphoblastic leukemia-associated antigens 257
Leukemia, myelogenous, acute in rat chemotherapeutic remissions in Wistar/Furth rat acute myelogenous leukemia

Neuraminidase *in vivo* behaviour neuraminidase-treated rabbit erythrocytes and reticulocytes 178
Neutrophilic leukaemia, Myelogenous leukaemia, chronic
Neutrophilic leukocytosis, Myelogenous leukaemia, chronic
Nitrate effect on haemoglobin congenital Heinz body haemolytic anaemia due to haemoglobin Perth in a *Negro* child seemingly aggravated by the high nitrate content of the water supply 135
Non-Hodgkin lymphomas Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211
Nonlipoid cryoglobulinaemia anti-γ-globulin activity DNA and antibodies to DNA in nonlipoid cryoglobulinaemia 344
Non-spherocytic haemolytic anaemia, Haemolytic anaemia
Non-spherocytic haemolytic anaemia, chronic, Erythrocyte membrane
Nuclear membrane system, Human eosinophils

Obituary Hedwig Löffler, Editor-in-Chief of *Acta haematologica* 366
Oncolytic drug, new Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211
Operations, major Postoperative platelet functions
Oral contraceptives, Pyruvate kinase deficiency

Paraproteinaemia B and T cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321
6-PGD (= 6-Phosphogluconate dehydrogenase) and G6PD (= Glucose-6-phosphate dehydrogenase) combined deficiency in *Hindu* boy 351
Peptichemio (= Synthetic multipeptide complex) A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211
Peripheral blood lymphocytes in systemic lupus erythematosus (Relation to activity) 199
Peripheral blood (lymphoid cells), B and T cell markers
Peroxidase, Lysosomes
Persantin® Dipyridamole
Philadelphia chromosome, Myelogenous leukaemia, chronic Y chromosome
Phosphatase, acid, Lysosomes
Phosphatase, alkaline, elevated (leukocytes), Myelogenous leukaemia, chronic
Phosphogluconate dehydrogenase 6-phosphogluconate dehydrogenase (= 6-PGD) and G6PD combined deficiency in *Hindu* boy 351
Plaquettes sanguines, Platelet( )
Plasma membrane system, Human eosinophils
Plasmacytoma case of lambda type tetramer Bence Jones proteinaemia 359
Platelet abnormalities, inherited, associated with low factor VIII activity in the same family 225
Platelet aggregation, circulating circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and lauric acids in guinea-pigs 87
Platelet antibodies circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135
Platelet calcium determination, simple and rapid method (Normal values and results in May-Hegglin anomaly) 168
Platelet function, postoperative, in patients on small subcutaneous doses of heparin 266

Males (bone marrow cells), v Y chromosome
Malignant lymphomas, v Lymphomas, malignant
Malignant tumors, v *Lymphocyte reactivity*
MAY HEGGLIN anomaly a simple and rapid platelet calcium determination (Normal values and results in MAY HEGGLIN anomaly) 168
Mediterranean anaemia, v Thalassaemia
Medulla ossium, v Bone marrow
Megaloblastic anaemia (vitamin B_{12} or folate deficiency) v Megaloblastic erythropoiesis
Megaloblastic erythropoiesis, electron microscope and high resolution autoradiographic studies 1
Membrane-bound immunoglobulins a simplified one-step procedure for the simultaneous determination of complement receptor lymphocytes and lymphocytes with membrane-bound immunoglobulins 74
Membrane of erythrocytes, v Erythrocyte membrane, Neuraminidase
Menadione sodium bisulphite, v Dipyridamole
Method a simple and rapid platelet calcium determination (Normal values and results in MAY-HEGGLIN anomaly) 168
– a simplified one-step procedure for the simultaneous determination of complement receptor lymphocytes and lympocytes with membrane-bound immunoglobulins 74
Microautoradiography v Autoradiography high resolution autoradiography
Microchromatography (DE 25) quantitation of Hb A_2 with DE-25 microchromatography in whole blood as screening test for β-thalassaemia heterozygotes 32
Microscope électronique, v Human B lymphocytes (leukaemic), Human eosinophils, Lysosomes, Megaloblastic erythropoiesis
Milz, v Spleen
Moelle osseuse, v Bone marrow
Mononucleosis infectiosa infectious nucleosis in HODGKIN's disease (case report) 152
Mosaicism pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354
Morbus HODGKIN's v HODGKIN's disease
Multipeptide complex, synthetic, v Peptichemio
Multiple myeloma, v B and T cell markers
Myelofibrosis, chronic idiopathic. A reversible disease? 171
Myelogenous leukaemia, chronic, with elevated leukocyte *alkaline phosphatase* positive indirect COOMBS's test neutrophilic leukocytosis and unusual cytogenetical findings 298
Myeloid leukaemia, chronic loss of the Y chromosome from bone marrow cells of males with myeloproliferative *disorders* (Report of two cases and review of the literature) 310
Myeloid metaplasia in the spleen histogenesis 81
Myeloma, multiple, v B and T cell markers
Myeloproliferative disorders loss of the Y chromosome from bone marrow cells of males with myeloproliferative disorders (Report of two cases and review of the literature) 310

Na, v Sodium
Nachruf HEINRICH LÜDIN, Editor-in-Chief of *Acta haematologica* 366
Nama child from *South West Africa (Namibia)* congenital Heinz body haemolytic anaemia due to haemoglobin Perth in a *Nama* child seemingly aggravated by the high nitrate content of the water supply 143
Namibia, v *Nama* child
Nekrolog HEINRICH LÜDIN, Editor-in-Chief of *Acta haematologica* 366

Neuraminidase *In vivo* behaviour neuraminidase-treated rabbit erythrocytes and reticulocytes 178

Neutrophilic leukaemia, Myelogenous leukaemia, chronic

Neutrophilic leukocytosis, Myelogenous leukaemia, chronic

Nitrate effect on haemoglobin congenital Heinz body haemolytic anaemia due to haemoglobin Perth in a Vaasa child seemingly aggravated by the high nitrate content of the water supply 135

Non-Hodgkin lymphomas Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211

Nonlupoid cryoglobulinaemia anti-γ-globulin activity DNA and antibodies to DNA in nonlupoid cryoglobulinaemia 344

Non-spherocytic haemolytic anaemias, Haemolytic anaemia

Non-spherocytic haemolytic anaemia, chronic, Erythrocyte membrane

Nuclear membrane system, Human eosinophils

Obituary Hermann Lüdin, Editor-in-Chief of *Acta haematologica* 366

Oncolytic drug, new Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211

Operations, major Postoperative platelet functions

Oral contraceptives, Pyruvate kinase deficiency

Paraproteinaemia B and T cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321

6-PGD (= 6-Phosphogluconate dehydrogenase) and G6PD (= Glucose-6-phosphate dehydrogenase) combined deficiency in *Hindu* boy 351

Peptichemio (= Synthetic multipeptide complex) A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211

Peripheral blood lymphocytes in systemic lupus erythematosus (Relation to activity) 199

Peripheral blood (lymphoid cells), B and T cell markers

Peroxidase, Lysosomes

Persantin®, Dipyridamole

Philadelphia chromosome, Myelogenous leukaemia, chronic Y chromosome

Phosphatase, acid, Lysosomes

Phosphatase, alkaline, elevated (leukocytes), Myelogenous leukaemia, chronic

Phosphogluconate dehydrogenase 6-phosphogluconate dehydrogenase (= 6-PGD) and G6PD combined deficiency in *Hindu* boy 351

Plaquettes sanguins, Platelet(s)

Plasma membrane system, Human eosinophils

Plasmacytoma case of lambda type tetramer Bence Jones proteinaemia 359

Platelet abnormalities, inherited, associated with low factor VIII activity in the same family 225

Platelet aggregation, circulating circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and linoic acids in guinea-pigs 87

Platelet antibodies circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135

Platelet calcium determination, simple and rapid method (Normal values and results in May-Hegglin anomaly) 168

Platelet function, postoperative, in patients on small subcutaneous doses of heparin 266

Platelet functions influence of cytostatics on some platelet functions *in vitro* (V Hydroxyurea) 242
Platelets (Recent advances in basic research and clinical aspects) Excerpta medica (1975) 127 (B)
Porphyrins, urinary erythrocyte δ-aminolaevulinic acid dehydratase, urinary porphyrins and porphyrin precursors in iron deficiency anaemia 305
Postoperative platelet function in patients on small subcutaneous doses of heparin 266
Potassium and sodium content of human red blood cells, effects of dipyridamole 193
Prednisolone Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-HODGKIN lymphomas 211
Preleukaemia acquired pyruvate kinase deficiency with haemolysis in preleukaemia 339
Procedure, v Method
Proceedings of the IXth Congress of the World Federation of Haemophilia (1974) Haemophilia 366 (B)
Protein study of folic-acid-binding protein in normal subjects 156
– v Paraproteinaemia
Pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354
Pyruvate kinase deficiency inherited erythrocyte pyruvate kinase deficiency Studies on 15 members of two related families 37
– – – red cell pyruvate kinase deficiency Adverse effect of oral contraceptives 116
Pyruvate kinase deficiency acquired, with haemolysis in preleukaemia 339

Quality control in haematology (Symposium of the International Committee for Standardization in Haematology) 128 (B)
Quinine, v Phosphogluconate dehydrogenase deficiency

Rabbit erythrocytes *in vivo* behaviour neuraminidase-treated rabbit erythrocytes and reticulocytes 178
Radio-carbon, v Cryoglobulinaemia
Radio-chrome v Transferrin-immune
Radio-iode, v Cryoglobulinaemia
Radio-iron, v Transferrin-immune.
Raster Elektronenmikroskop v electron microscopic study
Rats, v Spleen
Rats, Wistar/Furth rats chemotherapeutic remissions in Wistar/Furth rat acute myelogenous leukaemia A model for human acute myelogenous leukaemia 33
Red blood cells, v Erythrocyte(s), Human red blood cells
Red cell deformability v Erythrocyte membrane
Red cell pyruvate kinase deficiency Adverse effect of oral contraceptives 116
Red cell(s), v Erythrocyte(s), Human red blood cells
Reticulocytes of rabbits *in vivo* behaviour of neuraminidase-treated rabbit erythrocytes and reticulocytes 178
Reticulo-endothelial cells, v Myeloid metaplasia
Reticulum cell sarcoma, v Imidazole carboxamide
Reversibility of myelofibrosis chronic idiopathic myelofibrosis. A reversible disease? 171
Ribonucleic acid, v RNA
RNA (= Ribonucleic acid) synthesis, v Megaloblastic erythropoiesis
Rosette-forming cells, v Antileukaemic serum Cold agglutinins Immunoglobulins, membrane-bound Lupus erythematosus

Scanning electron microscopic study of leukaemic human B lymphocytes 247
Serum protein, v Folic-acid-binding protein
Sialic acid (membrane of erythrocytes), v Neuraminidase
Sideroblast score A sensitive indicator of

iron deficiency and hypoproliferative anaemia 24
Sideroblastic anaemia, pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354
Sideroblastic anaemia, idiopathic, acquired, refractory· Banded chromosome analysis in six patients 15
Sodium and potassium content of human red blood cells, effects of dipyridamole 193
South West Africa (Namibia) congenital Heinz body haemolytic anaemia due to haemoglobin Perth in a *Nama* child seemingly aggravated by the high nitrate content of the water supply 143
Spherocytosis, Non-spherocytic.
Spleen histogenesis of myeloid metaplasia in the spleen 81
Splenectomy Erythrocyte membrane, Myelofibrosis
Splenic haemopoiesis, Myeloid metaplasia
Staining, iron staining of bone marrow smears, Sideroblast score
Standardisation Quality control in haematology (Symposium of the International Committee for Standardisation in Haematology) 128 (B)
Statistische Auswertung, Delta-aminolaevulinic acid dehydratase Dipyridamole Haemoglobin A_2, quantitation Hydroxyurea Immunoglobulins, membrane-bound Lupus erythematosus Lymphocyte reactivity· Postoperative platelet function, Pyruvate kinase deficiency von WILLEBRAND factor
Submicroscopical structure, Ultrastructure
Symposium, Erythropoiesis, (congenital disorders) (B), Quality control. (B)
Synthetic multipeptide complex, Peptichemio
Systemic lupus erythematosus circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135
peripheral blood lymphocytes (Relation to activity) 199

T and B cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321
Tetrameric light chain case of lambda type tetramer Bence Jones proteinaemia 359
Tetraploid cell line in a girl with acute leukaemia 55
Thalassaemia effect of blood transfusion on the immune response of children with thalassaemia 65
Dipyridamole
Thalassaemia (α-thalassaemia) interaction of haemoglobin E with α-thalassaemia and haemoglobin Constant Spring 109
Thalassaemia (β-thalassaemia), heterozygotes, screening· quantitation of Hb A_2, with DE-25 microchromatography in whole blood as screening test for β-thalassaemia heterozygous 32
Therapy Hydroxyurea (tumors), Imidazole carboxamide (HODGKIN's disease), Peptichemio (lymphomas)
Thrombin, Antithrombin III
Thrombocytes, Platelet(s)
Thrombocytopenia circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and lauric acids in guinea-pigs 87
Thrombopathies, v Platelet abnormalities
Thrombosis, familial, due to antithrombin III deficiency in *Greek* family 47
Thrombosis, Platelet aggregates, circulating Platelet function, postoperative
Thymidine(^{3}H), Megaloblastic erythropoiesis
T lymphocytes B and T cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321
T lymphocytes, Antileukaemic serum,

Platelet functions *Influence of cytostatics on some platelet functions in vitro* (V Hydroxyurea) 242
Platelets (Recent advances in basic research and clinical aspects) Excerpta medica (1975) 127 (B)
Porphyrins, urinary erythrocyte δ-amino-laevulinic acid dehydratase, urinary porphyrins and porphyrin precursors in iron deficiency anaemia 305
Postoperative platelet function in patients on small subcutaneous doses of heparin 266
Potassium and sodium content of human red blood cells, effects of dipyridamole 193
Prednisolone Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-HODGKIN lymphomas 211
Preleukaemia acquired pyruvate kinase deficiency with haemolysis in preleukaemia 339
Procedure, v Method
Proceedings of the IXth Congress of the World Federation of Haemophilia (1974) Haemophilia 366 (B)
Protein study of folic-acid-binding protein in normal subjects 156
– v Paraproteinaemia
Pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism *in the bone marrow* 354
Pyruvate kinase deficiency inherited erythrocyte pyruvate kinase deficiency Studies on 15 members of two related families 37
– – – red cell pyruvate kinase deficiency Adverse effect of oral contraceptives 116
Pyruvate kinase deficiency acquired, with haemolysis in preleukaemia 339

Quality control in haematology (Symposium of the International Committee for Standardization in Haematology) 128 (B)
Quinine, v Phosphogluconate dehydrogenase deficiency

Rabbit erythrocytes *In vivo behaviour of* neuraminidase-treated rabbit erythrocytes and reticulocytes 178
Radio-carbon v Cryoglobulinaemia
Radio-chrome, v Transferrin-immune
Radio-iode, v Cryoglobulinaemia
Radio-iron, v Transferrin-immune
Raster Elektronenmikroskop, v Scanning electron microscopic study
Rate, v Spleen
Rats, Wistar/Furth rats chemotherapeutic remissions in Wistar/Furth rat acute myelogenous leukaemia A model for human acute myelogenous leukaemia 233
Red blood cells, v Erythrocyte(s), Human red blood cells
Red cell deformability v Erythrocyte membrane
Red cell pyruvate kinase deficiency Adverse effect of oral contraceptives 116
Red cell(s), v Erythrocyte(s), Human red blood cells
Reticulocytes of rabbits *in vivo* behaviour of neuraminidase-treated rabbit erythrocytes *and reticulocytes 178*
Reticulo-endothelial cells, v Myeloid metaplasia
Reticulum cell sarcoma, v Imidazole carboxamide
Reversibility of myelofibrosis chronic idiopathic myelofibrosis. A reversible disease? 171
Ribonucleic acid, v RNA
RNA (= Ribonucleic acid) synthesis, v Megaloblastic erythropoiesis
Rosette-forming cells, v Antileukaemic serum Cold agglutinins Immunoglobulins, membrane-bound Lupus erythematosus

Scanning electron microscopic study *of leukaemic human B lymphocytes* 247
Serum protein, v Folic-acid-binding protein
Sialic acid *(membrane of erythrocytes)*, v Neuraminidase
Sideroblast score A sensitive indicator of

Iron deficiency and hypoproliferative anaemia 24

Sideroblastic anaemia pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354

Sideroblastic anaemia, idiopathic, acquired, refractory Banded chromosome analysis in six patients 15

Sodium and potassium content of human red blood cells, effects of dipyridamole 193

South West Africa (Namibia) congenital Heinz body haemolytic anaemia due to haemoglobin Perth in Aawa child seemingly aggravated by the high nitrate content of the water supply 143

Spherocytosis, Non-spherocytic.

Spleen histogenesis of myeloid metaplasia in the spleen 81

Splenectomy Erythrocyte membrane, Myelofibrosis

Splenic haemopoiesis, Myeloid metaplasia

Staining, iron staining of bone marrow smears, Sideroblast score

Standardization Quality control in haematology (Symposium of the International Committee for Standardization in Haematology) 128 (B)

Statistische Auswertung, Delta-aminolaevulinic acid dehydratase Dipyridamole Haemoglobin A_2, quantitation Hydroxyurea Immunoglobulins, membrane-bound, Lupus erythematosus Lymphocyte reactivity Postoperative platelet function Pyruvate kinase deficiency von WILLEBRAND factor

Submicroscopical structure, Ultrastructure

Symposium, Erythropoesis, (congenital disorders) (B), Quality control. (B)

Synthetic multipeptide complex, Peptichemio

Systemic lupus erythematosus circulating anticoagulant against factor XII and platelet antibodies in systemic lupus erythematosus 135

– peripheral blood lymphocytes (Relation to activity) 199

T and B cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321

Tetrameric light chain case of lambda type tetramer Bence Jones proteinaemia 359

Tetraploid cell line in a girl with acute leukaemia 55

Thalassaemia; effect of blood transfusion on the immune response of children with thalassaemia 65

v Dipyridamole

Thalassaemia (α-thalassaemia) Interaction of haemoglobin E with α-thalassaemia and haemoglobin Constant Spring 109

Thalassaemia (β-thalassaemia), heterozygotes, screening quantitation of Hb A_2, with DE-25 microchromatography in whole blood as screening test for β-thalassaemia heterozygotes 32

Therapy Hydroxyurea (tumors), Imidazole carboxamide (HODGKIN's disease), Peptichemio (lymphomas)

Thrombin, v Antithrombin III

Thrombocytes, Platelet(s)

Thrombocytopenia circulating platelet aggregates and thrombocytopenia induced by intravenous infusions of arachidonic and lauric acids in guinea-pigs 87

Thrombopathies, Platelet abnormalities

Thrombosis, familial, due to antithrombin III deficiency in Greek family 47

Thrombosis, Platelet aggregates, circulating Platelet function, postoperative

Thymidine(³H), Megaloblastic erythropoesis

T lymphocytes B and T cell markers of bone marrow and peripheral blood lymphoid cells in patients with paraproteinaemia 321

T lymphocytes, Antileukaemic serum,

Platelet functions Influence of cytostatics on some platelet functions *in vitro* (V Hydroxyurea) 242
Platelets (Recent advances in basic research and clinical aspects) Excerpta medica (1975) 127 (B)
Porphyrins, urinary erythrocyte δ-amino-laevulinic acid dehydratase, urinary porphyrins and porphyrin precursors in iron deficiency anaemia 305
Postoperative platelet function in patients on small subcutaneous doses of heparin 266
Potassium and sodium content of human red blood cells, effects of dipyridamole 193
Prednisolone Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-HODGKIN lymphomas 211
Preleukaemia acquired pyruvate kinase deficiency with haemolysis in preleukaemia 339
Procedure, v Method
Proceedings of the IXth Congress of the World Federation of Haemophilia (1974) Haemophilia 366 (B)
Protein study of folic-acid-binding protein in normal subjects 156
– v Paraproteinaemia
Pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354
Pyruvate kinase deficiency inherited erythrocyte pyruvate kinase deficiency Studies on 15 members of two related families 37
– – – red cell pyruvate kinase deficiency Adverse effect of oral contraceptives 116
Pyruvate kinase deficiency acquired, with haemolysis in preleukaemia 339

Quality control in haematology (Symposium of the International Committee for Standardization in Haematology) 128 (B)
Quinine, v *Phosphogluconate dehydrogenase deficiency*

Rabbit erythrocytes *in vivo* behaviour of neuraminidase-treated rabbit erythrocytes and reticulocytes 178
Radio-carbon, v Cryoglobulinaemia
Radio-chrome, v Transferrin-immune
Radio-iode, v Cryoglobulinaemia
Radio-iron, v Transferrin-immune
Raster-Elektronenmikroskop, v Scanning electron microscopic study
Rate, v Spleen
Rats, Wistar/Furth rats chemotherapeutic remissions in Wistar/Furth rat acute myelogenous leukaemia A model for human acute myelogenous leukaemia 233
Red blood cells, v Erythrocyte(s), Human red blood cells
Red cell deformability v *Erythrocyte membrane*
Red cell pyruvate kinase deficiency Adverse *effect of oral contraceptives 116*
Red cell(s), v Erythrocyte(s), Human red blood cells
Reticulocytes of rabbits *in vivo* behaviour of neuraminidase-treated rabbit erythrocytes and reticulocytes 178
Reticulo-endothelial cells, v Myeloid metaplasia
Reticulum cell sarcoma, v Imidazole carboxamide
Reversibility of myelofibrosis chronic idiopathic myelofibrosis. A reversible disease? 171
Ribonucleic acid, v RNA
RNA (= Ribonucleic acid) synthesis, v Megaloblastic erythropoiesis
Rosette-forming cells, v *Antileukaemic* serum Cold agglutinins Immunoglobulins, membrane-bound Lupus *erythematosus*

Scanning electron microscopic study of leukaemic human B lymphocytes 247
Serum protein, v *Folic-acid-binding* protein
Sialic acid (membrane of erythrocytes), v Neuraminidase
Sideroblast score A sensitive indicator of

Index autorum ad Vol. 57

(B) = Book Reviews Buchbesprechungen Livres nouveaux

Acar S. Tekmalp, G. Özaoylu, Ş. Çevik, N., and Yaşar H. 188
Afeltra, A., Gandolfo, G. M.
Akın, F Pandolfi, F
Amadori, S Mandelli, F
Amoroso, A. Gandolfo, G M
Antonopoulos M v Stathakis, N. E.
Arapakis, G Economopoulos, T C.
Arnold, H Heimstädter V
Ascari, E., Baldini, C. L.
Austen, D E. G. and Rhymes, I. L. 127 (B)

Bacigalupo, A., Carella, A. M.
Basile, G Matthys, E., and Barbier P 290
Baldwin, C., Baldini, C. L.
Baldini, C. L. Riccardi, G. Sonzo, M. C. Ascari, E. Browell, A., and Baldini, C. 178
Barbier F Basile, G
Bar Sela, S., Rozenszajn, L. A.
Baumgartner-Stäubli, R. and Beck, E. A. 24
Beck, E A., Baumgartner-Stäubli, R.
Ben David, E., Rozenszajn, L. A.
Benoit, H., Kozaru, M.
Blingwell, A. G Death, S.
Boccadoro, F Gandolfo, G M
Botran, J Golomb, H. M., and Rowley J D. 15
Blume, K. G., Heimstädter V
Boccaccino, C. A., Greenberger J S.
Boschi, M., v Dachà, M.
Browell, A., Baldini, C. L.
Brittinger G Fischer K.
Bühler E. M., v DiLeo, P E.
Bush, V Wickramasinghe, S. N.

Canestrari, F Dachà, M.
Cao, A., Galanello, R.
Carella, A. M. Bacigalupo, A.; Santini, G. Giordano, D Damasio, E. E. Fusco, F A. Rossi, E., and Marmont, A. M. 211
Castinos, C v Kanakoudi-Tsakalidis, F
Çevik, N., Acar S.
Chalevelakis, G Lyberatos, C. Manopoulos, D. Pyrovolakis, J and Gardikas, C. 305
Charlow G. F Kendall, A. G.
Chvarda, E., Ponari, O.
Cohnen, G., Fischer K.
Cohen, M. M., Penchas, S.
Cohen, R., v Penchas, S.
Coster J F Lewis, S. M.
Courtis, M., v Gorst, D W
Crater D Rubinstein, L., and Menache, R. 168
Cronberg, S., Kubler, P

Dachà, M.; Canestrari, F Boschi, M. Rossi Ferrini, P L., and Fornaini, G. 37

Cold agglutinins, Lupus erythematosus
Transferrin-immune complex disease 96
Transformation of lymphocytes, v Lymphocyte reactivity
Transfusion, v Blood transfusion
Trisomy 8 pyridoxal-5-phosphate-resistant sideroblastic anaemia with trisomy 8 mosaicism in the bone marrow 354
Tritium, v Megaloblastic erythropoiesis
Tumor-associated antigens, v Leukaemia antigens
Tumor chemotherapy v Hydroxyurea, Imidazole carboxamide, Peptichemio
Tumoren, v Cancer patients

Ultrastructure electron microscope and high resolution autoradiographic studies of megaloblastic erythropoiesis *1*
– v Human leukaemic B lymphocytes, Human eosinophils, Lysosomes
Unstable haemoglobins, v Haemoglobin Perth, Haemoglobin Shepherds Bush
Uridine (^{3}H), v Megaloblastic erythropoiesis
Urinary porphyrins, v Porphyrins

Variant, v Glucose-6-phosphate dehydrogenase Hamburg, Glucose-6-phosphate dehydrogenase Velletri
Vincristine Peptichemio A new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas 211
Virus Epstein-Barr virus, v Infectious mononucleosis
Vitamin B_{12} deficiency v Megaloblastic erythropoiesis
von Willebrand factor antihaemophilic factor VIII activity factor VIII-related antigen and von Willebrand factor in hepatic cirrhosis 290

Waldenström's disease, v Cryoglobulinaemia
White human blood cells (Chediak Higashi syndrome) large granules and lysosomal fusion in human Chediak Higashi white blood cells 279
Willebrand v von Willebrand
World Federation of Haemophilia IX Congress, 1974 proceedings, v Haemophilia (B)

Y chromosome loss of the Y chromosome from bone marrow cells of males with myeloproliferative disorders (Report of two cases and review of the literature) 310

Zell v Cell
Zyto v Cyto

Lie Injo, L. E., Ganesan, J
Luzi, G., v Pandolfi, F
Lyberatos, C., Chalevelakis, G.

Mammon, Z., v Grinblat, J.
Mandelli, F Amadori, S. De Laurenzi, A. Kahn, A. Isacchi, G and Papa, G 121
Manoporios, D v Chalevelakis, G
Many A., Michalevicz, R.
Marmont, A. M., Carella, A. M.
Matthijs, E., Basle, G
Megha, A., Ponari, O
Mello, M. A., Galanello, R.
Menache, R., Creter D.
Meuret, G., Westerhausen, M.
Michalevicz, R. Many A., and Ramot, B. 199
Moeschlin, S 366
Moloney W C., Greenberger J. S.
Müller, HJ., DiLeo, P E.
Muroni, P Galanello, R.

Ng, T S., Ganesan, J

Obrecht, J.-P DiLeo, P E.
O'Connell, L., Creally J F
O'Gorman, D Creally J. F
Ooka, M. P Douglas, S. D
Özsoylu, Ş., Acar S.

Pandolfi, F Luzi, G. Tozzi, M. C., and Aiuti, F 257
Papa, G Mandelli, F
Papayannis, A G Economopoulos, T C.
Papayannis, A. G., Stathakis, N. E.
Pałczek, M., Kubler, P
Peake, I. R., Ulutin, O N.
Penchas, S Zlotnick, A. Cohen, R., and Cohen, M. M. 354
Pini, M., Ponari, O.
Protoufis, P Invernizzi, F
Ponari, O Civardi, E. Megha, A. Pini, M. Poti, R., and Dettori, A. G. 225
Poti, R., Ponari, O.
Pyrovolakis, J Chalevelakis, G.

Raffienbeul, D and Zbinden, G. 87
Ramot, B., Michalevicz, R.
Rhymes, I. L., v Austen, D E. G
Ribaldi, P S., v Invernizzi, F
Ricevuti, G., v Baldini, C. L.
Robinson, E., Haas, R.
Rossi, E., Carella, A. M.
Rossi Ferrini, P L., Dachà, M.
Rotter M., Winterleitner H.
Rowley J D Bitran, J
Rozenszajn, L. A. Ben David, E., and Bar Sela, S. 279
Rubinstein, L., Creter D.

Sakai, K., Komura, M.
Sansone, G. Sciarratta, G V Genova, R., Durbra, P D and Lehmann, H. 102
Santini, G v Carella, A. M.
Schröter W., Tillmann, W
Sciarratta, G. V., Sansone, G
Simon, N., v Haas, S.
Sosso, M. C., Baldini, C. L.
Speck, B., DiLeo, P E.
Spyrogiou, K., Kanakoudi-Tsakalidis, F
Stalder G R., DiLeo, P E.
Stathakis, N. E., Economopoulos, T C.
Stathakis, N. E. Papayannis, A. G. Antonopoulos, M., and Gardikas, C. 47
Sugimoto, H., Komura, M.
Szmikowski, W., Fischer K.
Szekely G., Kaleman, E.
Szot, S. J., Greenberger J S.

Tekinalp, G Acar, S.
Tillmann, W Gahr M. Labitzke, N., and Schröter W 162
Tozzi, M. C., Pandolfi, F
Tsaft, R., Kanakoudi-Tsakalidis, F

Uhl, N., Heimpel(?) V
Ulutin, O N. 127 (B)
Ulutin, O. N. and Peake, I. R. 366 (B)

Vettore, L. De Matteis, M. C., and Del Conte, G 196

Weatherall, D J 366 (B)
Westerhausen, M and Meuret, G 96

Damasio, E. E. v Carella A. M.
Darbre, P D v Sansone, G
Dash, S. and Bhagwat, A. G 351
Davidson, R. J L. and Lessels, S. E. 152
Delamore, I W v Gorst, D W
De Laurenzi, A. v Mandelli, F
Del Conte, G v Vettore, L.
De Matteis, M C. v Vettore, L.
Dettori, A. G v Ponari, O
DiLeo, P E. Müller HJ. Obrecht, J P Speck, B. Bühler E. M. and Stalder G R. 310
Dobozy A., v Husz, S.
Donner L. v Kiener P
Douglas, S. D and Ooka, M P 129

Economopoulos, T C. Papayannis, A. G Stathakis, N E. Arapakis, G., and Gardikas, C. 266
Endes, P v Kelemen, E.

Ferri, G M., v Gandolfo G M.
Fischer K. Cohnen, G Szaniawski W., and Brittinger G 247
Foadi, M. D. 55
Fornaini, G., v Dachà, M.
Fusco, F A., v Carella, A. M.

Gahr M., v Tillmann W
Galanello, R. Melis, M. A. Muroni, P., and Cao, A. 32
Gandolfo, G M Afeltra, A. Amoroso, A. Biancolella, F., and Ferri, G M. 135
Ganesan, J Lie Injo, L. E. Ng, T S., and George, R. 109
Gardikas, C. v Chalevelakis, G
Gardikas, C., v Economopoulos, T C.
Gardikas, C., v Stathakis, N. E.
Gavazzeni, G v Invernizzi, F
Genova, R. v Sansone G
George, R., v Ganesan, J
Giordano, D., v Carella, A. M
Golomb, H. M. v Bitran, J
Gorst, D W Courtis, M., and Delamore, I. W 156
Greally J F Whelan, C. A. O'Connell, L., and O'Gorman, D 206
Greenberger J S. Bocaccino, C. A. Szot, S. J., and Moloney W C. 233
Grinblat, J Mammon, Z. Lewitus, Z., *and Joshua, H.* 298
Grové, S. S. Jenkins, T Kamuzora, H. L., and Lehmann, H. 143

Haast, R. and Robinson, E. 331
Helmstädter V Arnold, H. Blume, K. G. Uhl, N and Hunstein, W 339
Hunstein, W., v Helmstädter V
Hunyadi, J., v Husz, S.
Husz, S. Krizsa, F Dobozy A. Hunyadi, J., and Simon, N. 321

Ibayashi, H., v Kozuru, M.
Invernizzi, F Pioltelli, P Gavazzeni, G Riboldi, P S., and Zanussi, C. 344
Isacchi, G v Mandelli, F
Iyengar B 81

Jenkins, T., v Grové, S. S.
Joshua, H. v Grinblat, J

Kahn, A. v Mandelli, F
Kamuzora, H. L., v Grové, S. S.
Kanakoudi-Tsakalidis, F Spyroglou, K. Tzafi, R. and Cassimos, C. 65
Kelemen, E. *Krasznai, G* Endes, *P.*, and Szinay G 171
Kendall, A. G., and Charlow G F 116
Kiener P and Donner L. 272
Knapp, W v Winterleitner H.
Kováčová, J v Kubisz, *P*
Kozuru, M. Benoki, H Sugimoto, H. Sakai, K., and *Ibayashi,* H. 359
Krasznai, G v Kelemen, E.
Krizsa, F., v Husz, S.
Kubisz, *P Pařízek, M Kováčová, J., and* Cronberg, S. 242

Labitzke, N., v Tillmann, W
Lehmann, H. v Grové, S. S.
Lehmann, H., v *Sansone,* G
Lessels, S. E., v Davidson R. J L.
Lewis, S. M and Coster J F 128 (B)
Lewitus, Z. v Grinblat, J

Whelan, C. A., v Greally J F
Wickramasinghe, S. N 128 (B)
Wickramasinghe, S. N and Bush V 1
Winterleitner H. Rotter M. and Krapp, W 74
Yaşar H., v Acar S.
Zanussi, C. v Invernizzi, F
Zbinden, G., v Raffenbeul, D
Zlotnick, A., v Penchas, S.